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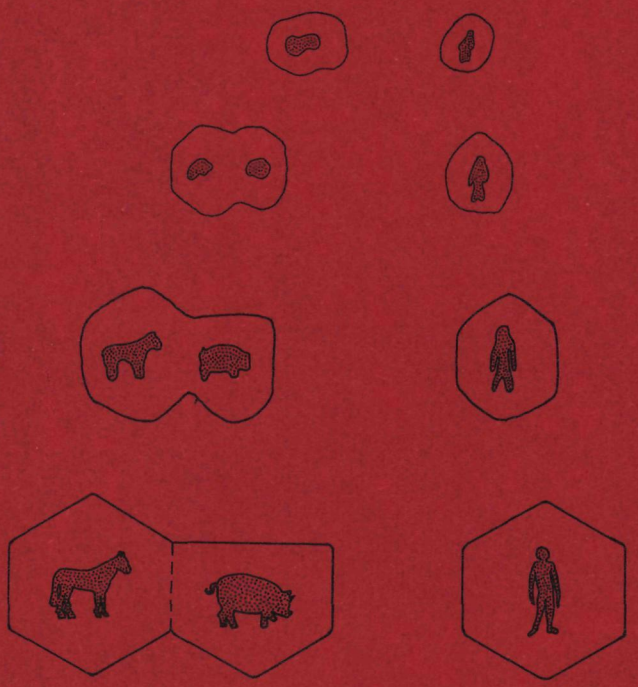
purine and pyrimidine metabolism in lymphoid cells of man and some other mammalian species



g.j. peters

PURINE AND PYRIMIDINE METABOLISM IN LYMPHOID CELLS OF MAN AND SOME OTHER MAMMALIAN SPECIES

Promotor: Prof. Dr. J.H. Veerkamp

PURINE AND PYRIMIDINE METABOLISM IN LYMPHOID CELLS OF MAN AND SOME OTHER MAMMALIAN SPECIES

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voor pap en mam

voor Marja

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Parts of the thesis are published or about to be published: Veerkamp et al. (403), Tax et al. (370), Peters & Veerkamp (267, 372) and Peters et al. (268-271, 273-276).

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Compounds

BSA, bovine serum albumin;
Con-A, Concanavalin-A;
d-, deoxy;
dNTP, deoxyribonucleoside triphosphate(s);
DHO, dihydro-orotate;
EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine;
Hepes, 2-{4-(2-hydroxyethyl)-1-piperazinyl}ethane sulfonic acid;
HPLC, High-performance liquid chromatography;
LPS, lipopolysaccharide;
MEMS, Minimum essential medium for suspension cultures;
PBL, peripheral blood lymphocytes;
PBS, phosphate buffered saline;
PEI, polyethyleneimine;
PHA, phytohemagglutinin;
Pi, inorganic phosphate;
PRPP, 5-phosphoribosyl 1-pyrophosphate;
PWM, pokeweed mitogen;
PPO, 2,5-diphenyloxazole;
POPOP, 2,2'-P-phenylene-bis (4-methyl-5-phenyl-oxazole);
ribose 1-P, ribose 1-phosphate;
ribose 5-P, ribose 5-phosphate;
RPMI 1640, Roswell Park Memorial Institute 1640 medium;
SAH, S-adenosyl-homocysteine;
SAM, S-adenosyl-methionine;
SCID, Severe Combined Immunodeficiency Disease;
SD, Standard Deviation; and
TCA, trichloro acetic acid.

Enzymes

Enzymes from purine metabolism (see Figs. 1.1, 1.3, 1.4, 1.7, 1.8, 5.1)

ADA, adenosine deaminase (EC 3.5.4.4.);
ADPase, adenosine triphosphatase;
AK, adenosine kinase (EC 2.7.1.20);
amido phosphoribosyltransferase (EC 2.4.2.14);
AMP deaminase, adenosine 5'-monophosphate deaminase (EC 3.5.4.6.);

AMP kinase, adenosine 5'-monophosphate kinase (EC 2.7.4.3.);
APRT, adenine phosphoribosyltransferase (EC 2.4.2.7.);
ATPase, adenosine triphosphatase;
deoxyadenosine kinase;
deoxyguanosine kinase;
GMP synthetase, guanosine 5'-monophosphate synthetase (EC 6.3.5.2.);
guanine deaminase (EC 3.5.4.3.);
HGPRT, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8.);
IMP dehydrogenase, inosine 5'-monophosphate dehydrogenase (EC 1.2.1.14.);
purine 5'-nucleotidase (EC 3.1.3.5.);
PNP, purine nucleoside phosphorylase (EC 2.4.2.1.);
PRPP synthetase, ATP : ribose 5-P pyrophosphotransferase (EC 2.7.6.1.);
SAH hydrolase, S-adenosyl-homocysteine hydrolase (EC 3.3.1.1.); and
xanthine oxidase, (EC 1.2.3.2.).

Enzymes from pyrimidine metabolism (See Figs. 1.2, 1.3)

ATC, aspartate transcarbamylase (EC 2.1.3.2.);
CPS II, carbamyl-phosphate synthetase (EC 2.7.2.9.);
cytidine kinase, (EC 2.7.1.48);
DHOase, dihydro-orotase (EC 3.5.2.3.);
DHO dehydrogenase, dihydro-orotate dehydrogenase (EC 1.3.3.1.);
deoxycytidine kinase, (EC 2.7.1.74.);
dCMP deaminase, deoxycytidylate deaminase;
OPRT, orotate phosphoribosyltransferase (EC 2.4.2.10);
ODC, orotidine 5'-monophosphate (EC 4.1.1.23.);
thymidine kinase, (EC 2.7.1.2.);
TMP synthetase, thymidine 5'-monophosphate synthetase (EC 2.1.1.45.);
uridine kinase, (EC 2.7.1.48.); and
uridine phosphorylase, (EC 2.4.2.3.).

Other enzymes (See Figs. 1.4, 1.5)

DNA pol I, DNA polymerase I;
glucose 6-P dehydrogenase;
luciferase;
phosphoribomutase;
ribonucleotide reductase;
TdT, terminal deoxynucleotidyl transferase;
transaldolase; and
transketolase.

Chapter 1

GENERAL INTRODUCTION

1.1. Introductory remarks

Studies with bacteria, fungi and several mammalian cells, cell lines and organs have given much insight in the mechanism of metabolism of purine and pyrimidine nucleotides. These studies showed that there are not only similarities but also differences between the several cell types, organs and species. Previous studies on purine and pyrimidine metabolism performed at this laboratory (see thesis Tax, 367) demonstrated that there are large differences in the nucleotide metabolism in red blood cells of various mammalian species. These studies also showed a close relationship between purine and pyrimidine metabolism in which PRPP plays a considerable role. The importance of normal regulation of metabolic routes is most obvious when one or more steps show an altered regulation. In Table 1.1 a summary is given of enzymes in purine and pyrimidine metabolism, that are associated with some kind of human disease. Details about these disorders can be found in the references.

The need to find the metabolic basis of the disturbances caused by ADA and PNP deficiencies in man leading to immune disorders, has given the impetus for this study. In the following sections the current knowledge on purine and pyrimidine metabolism and its regulation, especially in lymphocytes, will be reviewed. A short description of the immune system will be given before the immune disorders associated with aberrations in purine metabolism are discussed. The chapter will be concluded with the aim and scope of this study.

1.2. Purine metabolism

1.2.1. Purine nucleotide synthesis and interconversions

Purine nucleotides can be synthesized from several simple precursors like carbon dioxide and amino acids via the de novo pathway and via salvage pathways that use preformed nucleosides and bases that originate from the diet or are products of RNA and DNA breakdown (General reviews: 109, 113, 137, 196, 197, 281). Lymphocytes

Table 1.1. Primary disorders in purine and pyrimidine metabolism

Enzyme	Pathology	Reference
1. Deficiency or decreased activity		
HGPRT	Lesch-Nyhan syndrome	209
	X-linked gout	325
APRT	renal stones	51
ADA	SCID	122
PNP	severe T-cell dysfunction	123
Ecto-5'-nucleotidase	aggammaglobulinemia	93
AMP deaminase	muscle weakness	100
Adenylate kinase	hemolytic anemia	356
Xanthine oxidase	xanthinuria	83
PRPP synthetase	megaloblastic anemia, hypouricemia	405
Pyrimidine 5'-nucleotidase	hemolytic anemia	394
OPRT and/or ODC	orotic aciduria, megaloblastic anemia	169
2. Increased activity		
ADA	hemolytic anemia	395
PRPP synthetase	hyperuricemia	19

can also use preformed purines that were synthesized in the liver (244). A simple overall scheme of purine metabolism is presented in Fig. 1.1.

Purine de novo synthesis in lymphocytes has been studied by assay of several enzymes of the de novo pathway and by measurement of the incorporation of labelled precursors like {^{14}C}glycine and {^{14}C}formate into intermediates as formylglycinamide ribonucleotide and into nucleotides. Interpretation of the results of these experiments is usually difficult since the incorporated amount of radioactivity is mostly given in cpm and sometimes without the specific activity of the used precursor. However, from these experiments it is

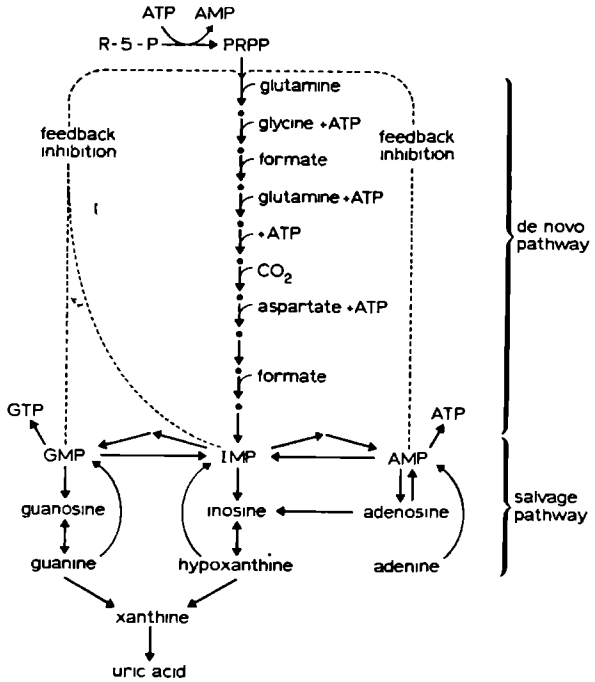


Fig. 1.1. De novo pathway and salvage pathways for the biosynthesis of purine nucleotides

clear that the overall rate of purine de novo synthesis in PBL of man and pig is very low and less than 5 pmol of glycine or formate incorporated into nucleotides per 10^6 cells/hr (168, 170, 191, 250, 343, 347). The rate of overall purine de novo synthesis in PBL is well below the activity of the first and rate-limiting enzyme in the purine de novo pathway, amido phosphoribosyltransferase (6, 17, 225, 408). The end products of purine de novo synthesis, the mono nucleotides, are involved in feedback regulation by inhibiting the amido phosphoribosyltransferase (17, 163, 299, 418). One substrate for the amido phosphoribosyltransferase, PRPP, is also a substrate for purine salvage pathways and pyrimidine de novo synthesis. Because of its importance, its regulating role in metabolism will be discussed in section 1.4.

An important purine salvage enzyme is HGPRT that catalyzes the synthesis of GMP and IMP from guanine and hypoxanthine, respectively. PRPP serves as the phosphoribosyl donor for these reactions and

for the synthesis of AMP from adenine, in a reaction catalyzed by APRT. Both enzymes have a considerable activity in lysates of human lymphocytes (18, 74, 84, 287, 320, 401, chapter 2). The substrates for HGPRT can be formed by the cell itself by degradation of nucleotides or can be taken up from plasma (422). No adenosine phosphorylase has been detected in mammalian cells and the only source of adenine for APRT is thought to be uptake by the intestine. However, in cultured cells it has been demonstrated that adenine can be formed in the polyamine pathway (189, 262), but this has not yet been demonstrated for PBL.

In intact cells incorporation of bases into nucleotides proceeds at a much lower rate (less than 5%) than in cell extracts at optimal substrate concentrations (135, 168, 239, 343). Adenine and guanine are incorporated into adenine and guanine nucleotides, respectively. Hypoxanthine is predominantly incorporated into adenine nucleotides (> 70%), the remainder into hypoxanthine and guanine nucleotides (239). These results and those obtained with other cell types (174, 339) indicate that generally the synthesis of GMP and AMP from IMP proceeds at a relatively rapid rate in intact cells, while the rates of the breakdown of AMP and GMP to IMP and the subsequent conversion of IMP to GMP and AMP, respectively, are very low. The activity of IMP dehydrogenase, a key enzyme in the regulation of GTP production (183) amounts to 14 nmol/hr per 10^6 cells (18). AMP kinase, necessary for the synthesis of ADP and ATP, has an activity ten times higher than the activity of AMP deaminase in human lymphocytes (102).

AMP can also be synthesized from adenosine by the action of AK. The enzyme from various sources has a MW of about 40 kD (7, 234, 424) and has a absolute requirement for Mg^{++} and ATP for its activity. The Km for adenosine is very low (about 1-5 µM) for purified preparations and crude extracts from various sources (8, 56, 182, 233, 234, chapter 5). The phosphorylation of deoxyadenosine can be catalyzed by adenosine kinase, but also by deoxycytidine kinase and a distinct deoxyadenosine kinase (3, 46, 49, 205, 216). The Km values for phosphorylation of deoxyadenosine are relatively high in various systems, including human lymphocytes (0.1 - 1 mM; 46, 109, 205, chapter 5). In human lymphocytes the activity of AK is about 1 nmol/hr per 10^6 cells (343, 401, chapter 5), the capacity of deoxyadenosine phosphorylation is lower (3; chapter 5). Deoxyguanosine phosphorylation is considered to be catalyzed by deoxycytidine kinase (46, 49, 181, 205) and a specific deoxyguanosine kinase (216). Phosphorylation of deoxyguanosine (253, 254, 314, chapter 8) has a higher activity

in human lymphocytes than that of deoxyadenosine (46, chapter 5).

1.2.2. Purine nucleoside metabolism

Adenosine and deoxyadenosine can be deaminated by ADA to inosine and deoxyinosine, respectively (reviewed by Fox & Kelley, 109; see Figs. 1.1, 1.7, 1.8, and 5.1). In lymphoid tissues ADA is present in one molecular form with a MW of 35 kD (159, 219, 399). A high MW form (MW 280 kD), that consists of the low MW form and a combining protein with a MW of 213 kD (80) is present in human lung, liver, intestine and kidney (219, 399). A 100 kD form is present in tissues of lower vertebrates (159) and man. The various forms have different kinetic properties (reviewed by Hirschhorn & Ratech, 159). The 35 kD enzyme has a comparable activity with adenosine and deoxyadenosine, a low Km value for both substrates (about 30 μM) and is completely inhibitable by EHNA and deoxycoformycin (80, 159, 294, 296). On the contrary, the 100 kD enzyme has a much higher activity with adenosine than with deoxyadenosine, a high Km value (about 1-3 mM) for adenosine and is not inhibited by EHNA and deoxycoformycin.

PNP catalyzes the phosphorylysis of the products of ADA to hypoxanthine, and of guanosine and deoxyguanosine to guanine. The enzyme has been purified from various sources and has shown to be a trimer with a total MW of 80-90 kD (121, 128, 176, 199, 256, 260, 317, 349, 412, 429). With the enzyme of most sources all four nucleosides show comparable kinetics and phosphorylysis rates. The Km values vary from 30-300 μM in the various preparations.

Large differences were noted in the activities of ADA (229, chapter 5) and PNP (34, chapter 5) in the lymphocytes of the various mammalian species. In human lymphocytes the activity of PNP is higher than that of ADA (250, 253, 399, chapter 5) and any inosine formed will rapidly be broken down to hypoxanthine. With intact lymphocytes adenosine can be deaminated and phosphorylated. At a low adenosine concentration (< 10 μM) phosphorylation is comparable to deamination, at higher concentrations deamination is higher (217, 287, 343). Since a guanosine and inosine kinase activity is not present or very low in mammalian tissues and lymphocytes (42, 265, chapter 8), these nucleosides are only phosphorylyzed. This also holds for deoxyguanosine in various cell types (174, chapter 8). Deoxyadenosine will be predominantly deaminated in PBL since the phosphorylating activity is very low and shows a high Km value for deoxyadenosine (chapter 5). Even with equine lymphocytes that have a very low ADA

activity, deoxyadenosine will be broken down to deoxyinosine (217) and subsequently to hypoxanthine. Xanthine oxidase, that catalyzes the further degradation of hypoxanthine to xanthine and uric acid, is not present in mammalian lymphocytes (248, 320).

The nucleosides adenosine, inosine and guanosine can be formed from their nucleotides in a reaction catalyzed by 5'-nucleotidase or a non-specific phosphatase (247). The activity of mono-5'-nucleotidase is comparable with IMP and AMP as substrates (373, 411, chapter 5). AMP deaminase has a considerable role in the breakdown of AMP in mammalian liver and muscle (398). It is also present in mammalian lymphocytes (101, 102, chapter 5) but the activity of adenylate kinase is much higher (102). An ecto-5'-nucleotidase has been found on mammalian lymphocytes (66, 93, 95, 373, 411, chapter 5). The AMP necessary for this reaction can be formed by an ecto-ADPase (341) and an ecto-ATPase (93) that are also present on lymphocytes. Some charateristics of the 5'-nucleotidase in lymphocytes were recently discussed by Uusitalo (393).

Except by dephosphorylation of AMP, adenosine can also be formed from SAH, that is formed in reactions where SAM serves as a methyl donor. A high concentration of adenosine or deoxyadenosine, as present in lymphocytes of patients with ADA-deficiency (206), can inhibit the enzyme SAH hydrolase (144, chapter 5). This can cause an accumulation of SAH that can inhibit methylation reactions (188).

1.2.3. Influence of mitogenic stimulation on purine metabolism

PHA-stimulation of lymphocytes induces a number of events in the cell (reviewed by Ling & Kay, 211) and is considered to be an in vitro function test of the ability of lymphocytes to recognize an antigen in vivo. The cell division is reflected by an enhanced purine synthesis de novo (168, 170, 250, 324, 343, 347). The requirement of purine biosynthesis de novo for lymphocyte response to mitogens was demonstrated by the marked suppression of this response by inhibitors of the purine de novo synthesis, azaserine and 6-mercaptopurine (5).

The activity of PRPP synthetase and amido phosphoribosyltransferase per mg protein is not increased at PHA-stimulation (6, 343, 408), but the concentration of PRPP is 3-10 fold elevated rapidly after the addition of PHA (74, 166, 343, chapter 3). This is probably due to an increased rate of the hexose monophosphate shunt (134, 231, 305, 313) resulting in a higher concentration of ribose 5-P,

one of the substrates of PRPP synthetase.

Purine salvage pathways also show a higher activity at PHA-stimulation. This is demonstrated most clearly by the increased incorporation into nucleotides of adenine, guanine and hypoxanthine (more than 5-fold) in human lymphocytes (168, 240, 287, 343). Adenosine incorporation is also increased, but only at a low concentration phosphorylation exceeds deamination (343). The increased incorporation of purine bases is accompanied by a higher cellular activity of HGPRT, APRT and AK (286, 287, 401) although no increase of HGPRT and APRT per mg protein was found (343). The activities of ADA and PNP show no significant change during PHA-stimulation (151, 152, 287, 335, 343, 401).

1.3. Pyrimidine metabolism

1.3.1. Synthesis and degradation of pyrimidine nucleotides

Synthesis of pyrimidine nucleotides can be mediated via de novo synthesis, that uses CO_2 and glutamine as precursors, and via a salvage pathway (Fig. 1.2; general reviews, 186, 208, 329). Since human lymphocytes are capable to incorporate small amounts of $^{14}CO_2$ (about 4 pmol per hr per 10^6 cells) into their pyrimidine nucleotides (178) all enzymes of the orotic acid pathway will be present. The first three enzymes of this pathway, CPS II, ATC and DHOase, copurify as one enxyme complex from the cytosol of most kinds of cells (reviewed by Jones, 186). In human lymphocytes it has only been demonstrated that CPS II and ATC form one enzyme complex (179). CPS has a very low activity in human lymphocytes (about 4 pmol carbamyl-phosphate synthesized per min per mg cell protein). The enzyme is inhibited by UTP, the end product of the orotic acid pathway and stimulated by PRPP (178, 359). It is considered to be the rate-limiting enzyme of the orotic acid pathway. The activity of ATC is 100-fold higher than that of CPS (179) and the activity of DHOase is 10% of that of ATC (214). The next enzyme in the orotic acid pathway, DHO dehydrogenase, has not yet been demonstrated in lymphocytes. In murine and rat liver it is located in the inner membrane of mitochondria (60, 198). Since lymphocytes have few mitochondria, activity of the enzyme could be rate-limiting for the novo pathway. The next two enzymes, OPRT and ODC, exist as an enzyme complex in most cell types (186, 329). The activity of OPRT in human lymphocytes is comparable to that of DHOase (214). In most kinds of cells the activity of ODC

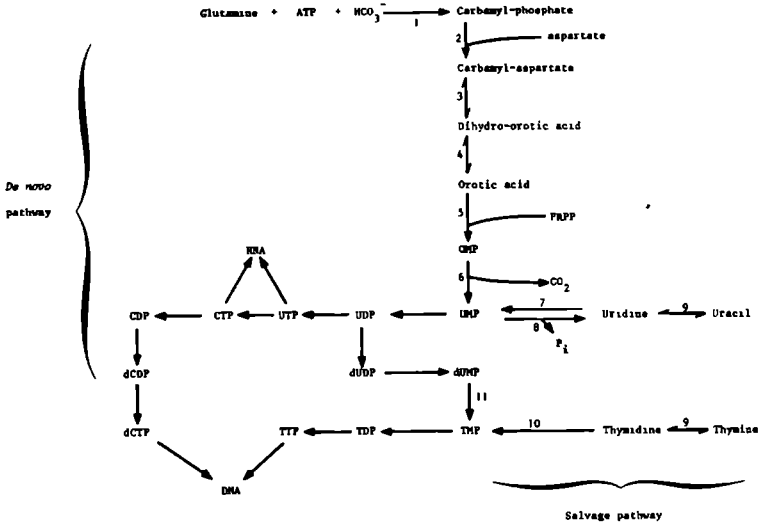


Fig. 1.2. De novo pathway and salvage pathway for the biosynthesis of pyrimidine ribonucleotides and deoxyribonucleotides. The enzymes catalyzing these conversions are: 1, CPS; 2, ATC; 3, DHOase; 4, DHO dehydrogenase; 5, OPRT; 6, ODC; 7, uridine kinase; 8, 5'-nucleotidase; 9, pyrimidine nucleoside phosphorylase; 10, thymidine kinase; and 11, TMP synthetase.

is about twice the activity of OPRT (114, 264, 363, chapter 2). OPRT can also be an important regulatory site of UMP synthesis (61, 164), especially when UTP levels are low.

The activity of uridine kinase in human PBL is comparable to that of OPRT (370). Other pyrimidine salvage pathway enzymes, thymidine kinase and deoxycytidine kinase are also present in human lymphocytes (243, 266), but the presence of specific salvage enzymes of the pyrimidine bases, uracil and thymine, has not yet been reported in lymphocytes. Degradation of pyrimidine nucleotides is catalyzed by a 5'-nucleotidase that shows substrate specificity in erythrocytes for UMP and CMP (260, 375). The products thymidine, cytidine and uridine can be phosphorylyzed by pyrimidine nucleoside phosphorylase to their bases thymine, cytosine and uracil, respectively. Uracil can also be formed by deamination of cytosine. With intact human lymphocytes uridine phosphorylysis exceeds phosphorylation, while with intact equine lymphocytes phosphorylysis is not measu-

rable (370). Further catabolism of thymine occurs via dihydrothymine and β-ureido-isobutyric acid to β-amino-butyric acid (392).

1.3.2. Influence of mitogenic stimulation on pyrimidine metabolism

At PHA-stimulation the rate of pyrimidine de novo synthesis increases. The 3-8 fold increase of bicarbonate incorporation (170, 178) is accompanied by a 3-fold increase of the activities of CPS and ATC (178, 179, 213), but no increase in the activities of DHOase, OPRT and ODC was found (214, chapter 4). Aspartate and orotic acid incorporation into nucleotides are only increased to a low extent at PHA-stimulation of human lymphocytes, while uridine incorporation increased more than 15-fold (213, chapter 7). This indicates that salvage pathways are more important at stimulation for the synthesis of pyrimidine nucleotides than the de novo pathway. Measurement of uridine and thymidine incorporation, that also increases more than 30-fold, are now established markers of PHA-stimulation (211), but agents that alter nucleoside transport or intracellular nucleotide pools can influence incorporation, independently of any effects on cellular proliferation (45, 89). The increase in pyrimidine nucleoside incorporation is accompanied by a more than 10-fold increase in the activities of uridine and cytidine kinase (180, 192, 213, chapter 4). The activities of the deoxynucleoside kinases, thymidine kinase and deoxycytidine kinase (165, 243, 266) and of deoxycytidylate deaminase also increase markedly (266). Thymidylate synthetase, that is responsible for the de novo synthesis of TMP by the methylation of dUMP, appears in human lymphocytes at PHA-stimulation (133). Thymine nucleotides synthesized via uridine kinase and thymidylate synthetase are more closely localized near the multi-enzyme complex of DNA replication than the thymine nucleotides derived from thymidine kinase (358). Unincorporated nucleotides are rapidly degraded (357). Pyrimidine nucleoside phosphorylase activity rapidly increases in Con-A stimulated murine lymphocytes (392) and PHA-stimulated equine and porcine lymphocytes (chapter 4).

1.4. Interaction of pyrimidine and purine metabolism

1.4.1. The role of PRPP

In intact cells purine and pyrimidine metabolism are no separate entities but interact with each other at several sites (revie-

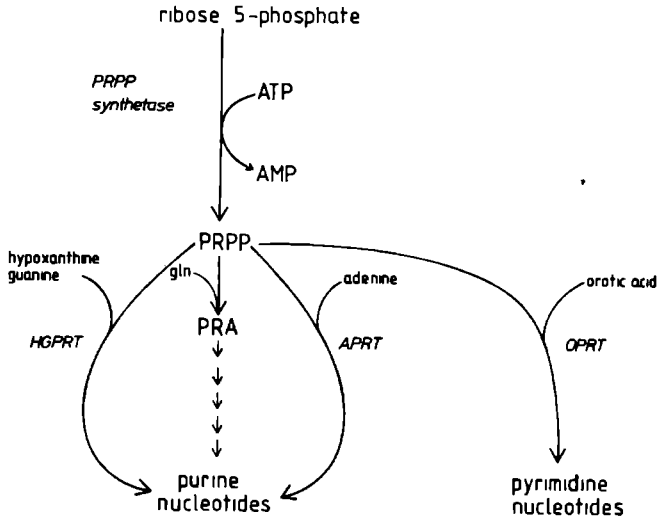


Fig. 1.3. Metabolism of PRPP. gln, glutamine; PRA, phosphoribosylamine.

wed by Tatibana, 360) and with the metabolism of amino acids and carbohydrates. The pentose unit is added to purine and pyrimidine precursors via PRPP by the action of several phosphoribosyltransferases; OPRT in pyrimidine metabolism, HGPRT, APRT and amido phosphoribosyltransferase in purine metabolism (Fig. 1.3). PRPP is also a substrate for many other phosphoribosyltransferases (reviewed by Fox & Kelley, 104 and Musick, 245), e.g. nicotinamide phosphoribosyltransferase, that is essential for pyridine nucleotide synthesis. Therefore its availability is crucial to cell metabolism.

PRPP synthesis from ATP and ribose 5-P is catalyzed by PRPP synthetase (reviewed by Becker et al., 21). Kinetics have been studied extensively for this enzyme from various sources (10, 105, 126, 306, 355, 417, chapter 3). AMP, the other product of the PRPP synthetase reaction, and ADP are powerful inhibitors of the enzyme and to a lower extent also pyrimidine and guanine nucleotides. Inorganic phosphate is an essential allosteric activator for PRPP synthetase activity (14, 147, 289). ATP shows substrate inhibition (77, 306, chapter 3). Mutant PRPP synthetases with aberrant kinetics were found in erythrocytes from gouty patients (19, 251, 346). The hyperactivity probably contributes to the hyperuricemia.

Ribose 5-P, the precursor for PRPP, is a product of the pentose

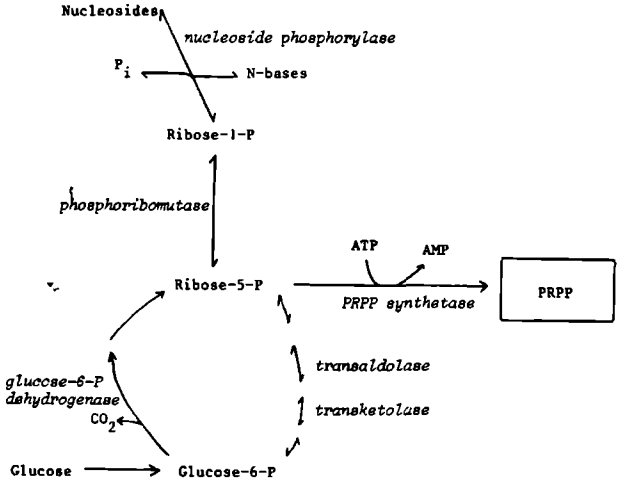


Fig. 1.4. Metabolic routes leading to the synthesis of PRPP

phosphate pathway, as well of the oxidative as of the non-oxidative branch (Fig. 1.4). In fibroblasts the non-oxidative branch seems to be predominant for ribose 5-P synthesis (290), but in erythrocytes stimulation of the oxidative branch results in an increase of the PRPP concentration (425). It is not clear what branch is responsible for ribose 5-P synthesis in peripheral and stimulated lymphocytes. At PHA-stimulation of lymphocytes a higher [1-^{14}C]glucose oxidation reflects a higher activity of the oxidative branch of the pentose phosphate pathway (134, 231, 305). At PHA-stimulation also an elevation of the PRPP concentration is observed (74, 166, 343, chapter 3). However, both phenomena have not yet been studied simultaneously. Ah alternative source for ribose 5-P may be the ribose 1-P that is formed by the phosphorylysis of ribonucleosides (336). By the action of phosphoribomutase ribose 1-P can be converted to ribose 5-P.

1.4.2. Interaction of nucleotides

Nucleotides are necessary in balanced amounts for the synthesis of RNA and DNA. Ribonucleotide availability is controlled by feedback inhibition of the end products and their concentrations mostly show little fluctuations under normal conditions. ATP has the highest concentration of all nucleotides in most cell types. In human lympho-

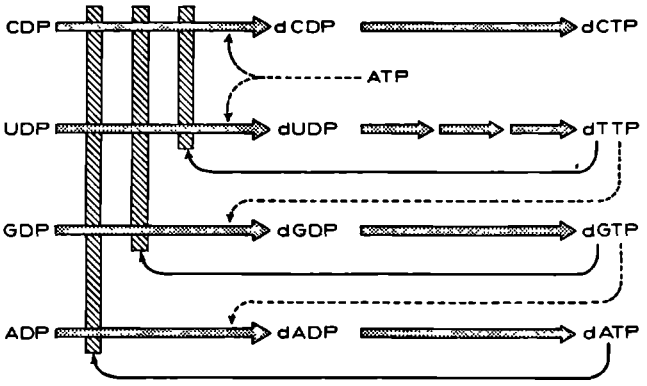


Fig. 1.5. Allosteric regulation of ribonucleotide reduction. Solid lines represent inhibition of the reduction of the ribodinucleotides indicated by the cross-hatched bars. Broken lines represent activation of enzyme activity for the indicated compounds (Modified from 281 and 301).

cytes its concentration is about 600 pmol/10^6 cells (79, 246, 318, 321, 416, chapter 9). ATP is not only an energy donor for many anabolic routes but it also acts as a metabolic regulator for ribonucleotide reductase (238). This enzyme catalyzes the reduction of CDP, UDP, GDP and ADP to their deoxycompounds. dNTP act in these reactions as allosteric effectors (301). The complex regulation of this enzyme appears to be different in several cell types (173). A simplified scheme of its regulation, that seems to hold for most cell types is presented in Fig. 1.5. Regulation includes feedback inhibition by dATP of the reduction of all ribodinucleotides, inhibition by dGTP of the reduction of GDP, CDP and UDP, and inhibition by TTP of the reduction of UDP and CDP; stimulation by ATP of the reduction of pyrimidine ribodinucleotides and by TTP of the GDP reduction. The concentrations of the dNTP are very low in peripheral human lymphocytes (0.1 to 10 pmol/10^6 cells) being the lowest for dGTP (175, 195, 361, 384, 385, chapter 9). When the cells enter the S-phase (e.g. at PHA-stimulation) an increase of the activity of DNA polymerase is paralleled with an increase in the concentration of some dNTP (242, 384, 385).

1.5. Some aspects of the immune system

Lymphocytes are small circulating cells (diameter about 10 µm), that play an important role in immune function. Knowledge of the ontogeny and the function of the immune system considerably increased in the last decade and several reviews and theses on this subject have recently been published (see e.g. 27, 72, 82, 98, 204, 304, 402). These reviews form the basis of a short summary of some aspects of immunology that are necessary for proper understanding of the following sections.

Lymphocytes and all other blood cells are formed from a common stem cell located in the fetal liver and the bone marrow. The lymphocytes differentiate further into T- and B-cells and some cells that bear no T- or B-cell markers (Fig. 1.6). B-lymphocytes are responsible for the humoral immunity (production of antibodies against most bacteria and other non-self cells). In mammals B-cells are probably formed in the bone-marrow or in lymphoid tissues like the lymph nodes, tonsils and the lamina propia of the gut (82). T-cells are responsible for the cellular immunity (against viri and fungi infections and also some bacteria, tumors and transplantates).

The thymus serves as a site for T-cell differentiation. This gland is a bilobed organ located just above the heart. Neonatal thymectomy (removal of the thymus) results in severe depletion of the lymphocyte population and serious immunological defects of the mature animal. Adult thymectomy generally does not accelerate the decline in immunocompetence that is normally found with ageing. The thymus plays an important role in the ontogeny of the immune system, especially in the period just before delivery until the time that the involution of the thymus begins. Involution is characterized by a decline in weight of the total thymus, an increase in the proportion of fat and the depletion of cortical lymphoid areas. Involution of the thymus in man starts after puberty and with rats and mice after one month.

Several subsets of T-cells originate from the thymus. Undifferentiated prothymocytes that lack all the T-cell characteristics, enter the thymus and differentiate/mature initially in the outer part of the thymus, the thymic cortex. Then the cells partially move to the thymic medulla where they further mature. During their stay in the thymus, cells develop distinct changes of cell surface markers and functional properties. This process is induced by several thymic hormones.

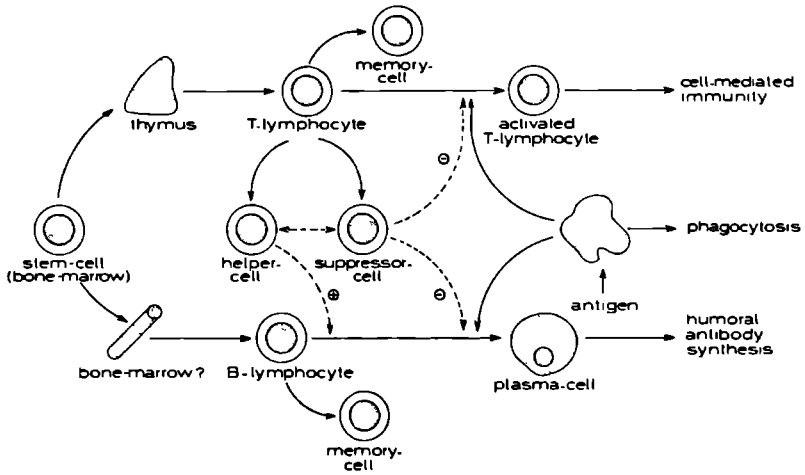


Fig. 1.6. Ontogeny and regulation of the cellular immune system (modified from 304)

Fig. 1.6 shows the interactions of several T-cell subsets and B-cells in order to mediate cellular and humoral immune response. Macrophages play an important role in the immune response, because they first recognize an antigen. The cell bearing the antigen can be destroyed by phagocytosis by the macrophage itself or the macrophage presents the antigen to either a T-lymphocyte or a B-lymphocyte. When it is presented directly to a B-cell, this cell can proliferate to a plasma cell that produces specific antibodies. Some of these cells proliferate to memory B-cells that facilitate recognition and destruction of the antigen in a recurrent infection. When the antigen is presented to a T-cell, several possibilities exist. When a memory T-cell recognizes the antigen it starts to grow and produce lymphokines that help to make harmless the cells bearing the antigen. A cytotoxic T-lymphocyte (a killer cell) will attach to the target cell and destroy it. The macrophage can also present the antigen to a T-helper cell, that activates B-cells to antibody production. T-suppressor cells can inhibit the antibody production. In vitro these processes are studied by activating B-cell with mitogens like PWM or LPS and T-cells with PHA or Con-A. Most mitogenic compounds are isolated from plants (PWM, PHA, Con-A) or bacteria (LPS).

Aberrations in the populations of B and/or T cells or their sub-

sets can results in severe disturbances of the immune response. This may happen in immune diseases and leukemia.

1.6. Some aspects of nucleoside and nucleotide metabolism in lymphoid tissues

There is acqumulating evidence that some enzymes involved in purine metabolism can be used as markers for different lymphoid cells. The most pronounced example is TdT, an enzyme that has the capacity to add deoxyribonucleotides to any 3'-OH-terminated segment of DNA without template direction (31, 32). TdT^+-cells first appear in the thymus during late fetal life. With adult animals the majority of immature cortical thymocytes show TdT activity. Some TdT^+-cells appear in liver, lung, spleen and bone marrow of rats within a few weeks after birth (316), but disappear with maturation. Only in the thymus and bone-marrow TdT^+-cells are found with adult mammals, including man (31). The presence of this enzyme is considered as a marker of immature lymphocytes.

ADA also shows a specific tissue distribution. With several mammals including man, it has the highest activity in lymphoid tissues (1, 46, 153, 229, 379, 380, 382), although the ADA activity in intestinal tissues of some animals is higher or comparable (39, 372, 380). In human lymphoid tissues ADA activity is highest in thymus, with a lower activity in spleen and lymph nodes (1). Barton et al. (15, 16) and Chechick et al. (59) demonstrated that the high ADA activity in rat and human thymus is predominantly located in the cortical thymocytes. ADA activity in medullary rat thymocytes may be similar to that in peripheral T-lymphocytes (15, 16), but no significant difference in activity of ADA between human T- and B-cells has been found (250, 253, 335, 401). Only MacDermott et al. (220) found a significantly higher ADA activity in human T-lymphocytes than in B-lymphocytes.

In contrast to the ADA activity no lymphocyte predominance is found for PNP activity (46). Thymus and spleen had low and intermediate activities, respectively, in comparison to other human and murine tissues (46, 372). Barton et al. (15, 16) postulated an inverse relationship between ADA and PNP in rat lymphoid cell populations. Thymocytes had the highest ADA activity and the lowest PNP activity, whereas spleen and bone marrow had the lowest ADA activity (much lo- than thymocytes) and the highest PNP activity (much higher than thymocytes). Specific stages of T-cell differentiation may be characte-

rized by the levels of the two enzymes according to Barton et al. (15, 16), but this does not appear to hold for changes in enzyme activity during life in lymphoid cells of rat and mice (322, chapter 10). Sheep and pig also show no inverse relationship in their ADA and PNP activities at comparison of thymocytes, splenocytes and PBL (chapter 11). The lower PNP activity in thymus in comparison to in spleen is not reflected by a lower PNP activity in T-cells, since several authors found a higher PNP activity in peripheral T-cells than in B-cells (33, 220, 250, 314).

The activity of ecto-5'-nucleotidase appears to be higher in B-cells than in T-cells (309, 310, 373), but a similar activity for both cell types has also been reported (66, 95, 96). However, with lymphoid tissues the lowest activity was found in the thymus (86, 393). Immature thymocytes appear to have the lowest ecto-5'-nucleotidase activity. The activity of this enzyme is considered to be a marker of B- and T-cell maturation (36). An age-related fall in T- and B-lymphocyte ecto-5'-nucleotidase was found by Boss et al. (37). The fall follows the decline in immune system function, that is known to occur with age (194).

The activity of AK is comparable in most tissues, lymphoid and non-lymphoid (3, 46), but that of deoxycytidine kinase is, as well with deoxyadenosine, deoxyguanosine or deoxycytidine, highest in human thymus (46, 49). Human T- and B-cells differ in their ability to use deoxycytidine as a precursor for DNA synthesis (4); B-cells do not incorporate deoxycytidine into DNA under circumstances where thymidine is incorporated. As T-cells mature there is a decrease in the activity of deoxycytidine kinase (67). B-cells have even a slightly higher activity of this enzyme than peripheral T-lymphocytes. In adult rats the activity of thymidine kinase and thymidylate synthetase is much higher in thymus than in other tissues (148). Thymidine kinase activity was much higher in thymus than in other tissues (148). Thymidine kinase activity was much higher in thymus and spleen before and immediately after birth. The activities of APRT and AK are comparable in T- and B-cells, that of HPRT is higher in T-cells and that of PRPP synthetase higher in B-cells (250).

1.7. Aberrations in the immune system and purine metabolism

The relationship between disturbances of purine metabolism and of immune function is illustrated in Fig. 1.7. The first two cases were reported in 1972 by Giblett et al., who described two patients

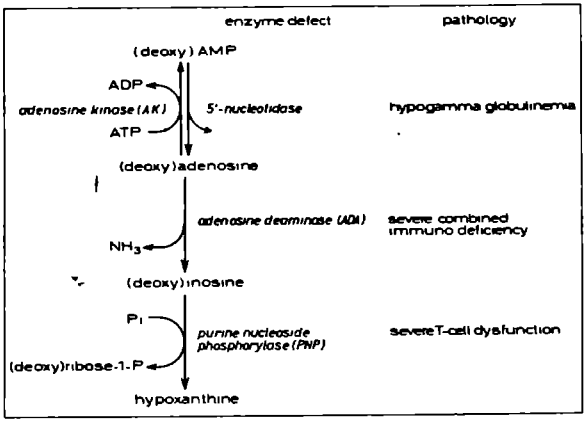


Fig. 1.7. Relationship between deficiencies in enzymes of purine metabolism and the pathology of the immune system

with SCID, that had no detectable ADA activity in their erythrocytes. In 1975 they described a patient with severe T-cell dysfunction with no detectable PNP activity in the erythrocytes. These relationships appeared to be causal in contrast to the ecto-5'-nucleotidase deficiency that was reported in several cases of agammaglobulinemia (93, 187).

All cases of SCID, including those with ADA deficiency, are associated with a severe impairment of the T- and B-cell mediated immune system (161), clinically associated with a severe lymphopenia and a relative and absolute decrease in T- and B-cell numbers. Immunoglobulin synthesis is severely depressed and the remaining lymphocytes show no or a very low response to PHA, PWM and Con-A stimulation (91, 311, 381). The defect in the B-cell system is responsible for lack of defense against exotoxin-secreting bacteria, viri, fungi and protozoa. Post-mortem findings showed a quantitative and qualitative depletion in the lymphatic tissues. In most cases the thymus is not present or it is very small and there is no clear distinction between cortex and medulla (154).

Inherited ADA deficiency appeared to be the cause of SCID in about 30-50% of the reported cases (161). The ADA activity was low in erythrocytes, but also in other tissues (153). The deficiency was limited to the low MW enzyme; the ADA activity in serum of an ADA-deficient child appeared to be due to the high MW enzyme (294).

In most cases ADA deficiency was established in erythrocytes. In about 10 cases red cell ADA does not appear to be a good parameter since some children with erythrocyte ADA deficiency did not show immunodeficieny (35, 155, 185), probably because in their lymphocytes a certain ADA activity was present. Furthermore some patients with SCID had even an elevated red cell ADA activity, whereas the activity in white blood cells and fibroblasts was normal (62). In addition to the above described characteristics ADA deficiency associated with SCID sometimes shows skeletal and neurological abnormalities (158). Although in horses SCID is also found with the same characteristics as with ADA deficiency in man (230) a relatioship with purine metabolism is not clear.

Inherited PNP deficiency associated with severe T-cell dysfunction was first descibed in 1975 (123) in a five year-old girl that showed recurrent infections. This patient and other patients described (25, 44, 119, 332) show a severe lymphopenia and a deficient T-lymphocyte blastogenic response. Deficiency was established in erythrocytes and fibroblasts and only in one case also in lymphocytes (315). Molecular heterogeneity was observed in different families with enzyme-deficient members (25, 256, 420). Humoral immunity was normal as appeared from normal amounts of B-lymphocytes and normal to elevated levels of immunoglobulins. The excessive and abnormal antibody production found with some patients suggest disturbances in suppressor T-cell function (25, 119). The patients show recurrent respiratory infections, but received most immunizations without complications. Post-mortem findings and X-ray examinations showed that the thymus was barely recognizable and consisted mainly of adipose tissue.

Agammaglobulinemia is characterized by the absence of B-cells in the peripheral blood and very low immunoglobulin levels. In many patients with agammaglobulinemia a very low activity of ecto-5'-nucleotidase is present on lymphocytes (36, 93, 96, 187, 373). The deficiency is more pronounced in B-cells that normally show higher levels of ecto-5'-nucleotidase than T-cells. Until now there is no evidence that this enzyme deficiency is responsible for the observed immune disorder, but evidence increases that it is only a marker of immature T- and B-cells (36, 37, 66, 96). The decrease of activity is associated with a reduction in number of cells containing 5'-nucleotidase (298, 373).

The most succesfull therapy for SCID with or without ADA deficiency is bone-marrow transplantation (161). For patients with some

residual ADA activity in their lymphocytes transfusions of frozen irradiated erythrocytes appear to give partial restoration of their immune function (91, 158, 175, 279, 280, 311, 319, 381, 430). An increased response to mitogens, an increased number of lymphocytes and higher levels of serum immunoglobulins were found thereafter. With some patients with PNP deficiency infusion with irradiated frozen erythrocytes also resulted in partial restoration of the immunological function (303, 348).

1.8. The metabolic basis for ADA and PNP deficiency

The metabolic basis for ADA and PNP deficiency leading to immuno disorders was investigated by studying the lymphocytes and erythrocytes of patients with ADA or PNP deficiency before and after enzyme replacement therapy and from studies with several cell lines that showed little or no ADA or PNP activity or that were treated with ADA inhibitors like EHNA or deoxycoformycin. Several reviews on this subject have been published (109, 113, 124, 138, 139, 160, 227, 237, 258, 281, 288, 327). In Table 1.2 a summary of the various hypotheses is given concerning the mechanisms that in ADA or PNP deficiency can lead to the expression of immuno disorders. These hypotheses will be discussed in connection with biochemical observations and investigations.

Deficiency of ADA leads to accumulation of its substrates, adenosine and deoxyadenosine, in serum, plasma, erythrocytes and urine of patients (64, 158, 206, 333). Deficiency of PNP leads to accumulation of inosine, guanosine and their deoxycompounds (63, 256, 333, 348, 351, 420) and to a decrease of their products hypoxanthine and uric acid. Since all nucleosides share the same transport system (263, 277) accumulated nucleosides could inhibit the uptake of the other nucleosides, causing a depletion of their nucleotides. However, until now no direct evidence for this hypothesis has been found.

Evidence for the second hypothesis has recently been given by Tritsch & Niswander (377, 378) who observed a relationship between ADA activity and xanthine oxidase-catalyzed superoxide formation in rat macrophages. They suggested that the absence of substrate for xanthine oxidase in ADA deficiency is partly responsible for the impaired immune response. Since adenosine can serve as an energy donor to lymphocytes by its ribose moiety, deficiency of ADA may lead to disturbance of the energy balance (252).

Accumulation of nucleosides and subsequently their nucleotides

Table 1.2. Possible mechanisms that can lead to immune disorders in ADA or PNP deficiency

1. Inhibition of uptake of other nucleosides
2. Deficiency of products (inosine, hypoxanthine, ribose 1-P)
3. Intracellular accumulation of (deoxy)nucleosides or nucleotides, which may cause;
   a. inhibition of PRPP synthesis,
   b. inhibition of pyrimidine nucleotide synthesis de novo,
   c. disturbance of the energy charge,
   d. inhibition of ribonucleotide reductase,
   e. inhibition of methylation reactions,
   f. other effects (possibly mediated by cAMP).

can affect various metabolic conversions; some possibilities are summarized in the third hypothesis. In addition to nucleosides, nucleotides also accumulate in ADA and PNP deficiency. In ADA deficiency elevated concentrations of dATP were found in erythrocytes and lymphocytes of several patients (64, 69, 85, 158, 175, 430). In PNP deficiency elevated concentrations of dGTP were found in erythrocytes of two patients (65, 352). A decreased ATP/ADP ratio was found in lymphocytes of an ADA deficient patient (318). In vivo inhibition of ADA with deoxycoformycin leaded to ATP depletion and dATP accumulation in erythrocytes of man and mouse (331, 340). The observed ATP depletion may lower the energy charge leading to inhibition of various anabolic pathways, e.g. inhibition of PRPP synthesis (chapter 3). The nucleotides AMP and dAMP inhibit PRPP synthetase (see section 1.4.1). Established cell lines of fibroblastic and lymphoid origin die of pyrimidine nucleotide starvation in the presence of 10-100 μM adenosine (125, 128, 177). This starvation is not observed in a Chinese hamster lung fibroblast line that was AK-deficient (80). Furthermore adenosine blocks pyrimidine synthesis in erythrocytes and fibroblasts by decrease of PRPP concentration (110). However, an increase of pyrimidine nucleotides was found in lymphocytes of an ADA-deficient patient (318). Uridine does not prevent the toxic effects of adenosine in ADA-deficient fibroblasts (23, 110) and in stimulated ADA-deficient or ADA-inhibited cells (45, 279) and at a high adenosine concentration (202). Therefore a block in pyrimidine synthesis does not seem to be the basis for this immune disorder. In PNP deficiency depletion of pyrimidines and PRPP has not been

excluded as a possible mechanism of disturbance of cell function (94, 110).

One of the most probable mechanisms explaining the effects of ADA and PNP deficiency leading to immune dysfunction is inhibition of ribonucleotide reductase by dATP or dGTP, leading to depletion of guanine and/or pyrimidine dNTP (Fig. 1.5). Since the adequate supply of dNTP is disturbed, the cell growth will be inhibited. In several cell types simulation of ADA deficiency with ADA inhibitors like EHNA and deoxycoformycin and addition of deoxyadenosine, resulted in growth inhibition, inhibition of DNA and RNA synthesis and accumulation of dATP (47, 111, 145, 235, 388, chapter 9). T-cell lines appeared to be more susceptible to deoxyadenosine than B-cell lines (47, 421). Deoxyguanosine toxicity was accompanied by an increase in dGTP concentration with human lymphoblasts (120, 235, 414), mouse lymphoma cells (53, 127, 389) and human lymphocytes (362) and thymocytes (67, 195). T-cells appeared to be more susceptible to deoxyguanosine than B-cells (67, 120, 235). In the mouse suppressor T-cell development was inhibited by deoxyguanosine (87). Cohen et al. (68) suggested that not only dGTP accumulation but also GTP depletion would inhibit DNA synthesis. However, inhibition of ribonucleotide reductase can not form the only explanation for the disturbances in lymphoid cells, since deoxyadenosine and deoxyguanosine also cause dATP and dGTP accumulation, respectively, in non-stimulated lymphocytes and non-dividing thymocytes and kill these cells (195). Furthermore adenosine and deoxyadenosine show the most pronounced effects on growth of lymphoid cells when added at the first day of culture (374, 386), when the cells are still in the inactivated state and the proliferation and DNA synthesis have not yet started. Deoxyadenosine plus EHNA in contrast to either deoxyguanosine or hydroxyurea, produced little differential effect on the incorporation of uridine and thymidine into DNA of a T-lymphoblast line (414). This also suggests another or additional mechanism to ribonucleotide reductase inhibition for deoxadenosine toxicity. High concentrations of dGDP cause a feedback inhibition of amido phosphoribosyltransferase (149). Incubation of T-lymphoma cells with deoxyguanosine resulted in a depletion of ADP and ATP (53).

There is also evidence that adenosine and deoxyadenosine have not to be phosphorylated to cause inhibition of cell function (142). Under physiological conditions adenosine can be formed from SAH, a product of methylation reactions where SAM is a substrate (Fig. 1.8). In ADA deficiency adenosine accumulates and reverses the physiologi-

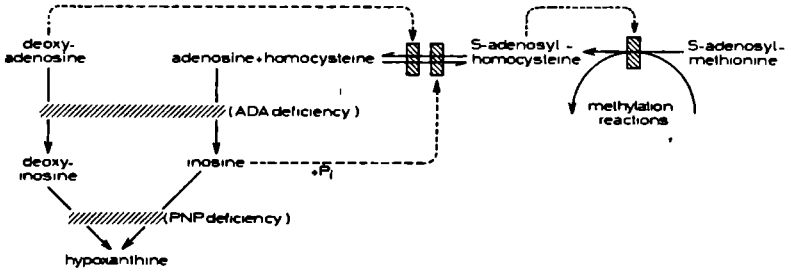


Fig. 1.8. Proposed mechanism for the inhibition of methylation reactions in ADA and PNP deficiencies. Cross-hatched bars represent the inhibitions caused by accumulation of adenosine, deoxyadenosine or inosine (modified from 281).

cal direction (145). The accumulated SAH will inhibit methylation reactions. The deoxyadenosine that accumulates in ADA deficiency, is able to inactivate SAH hydrolase (143) thus causing an accumulation of SAH. This inactivation is irreversible and in erythrocytes of patients with ADA deficiency no activity of SAH hydrolase is detectable (144, 190). In lymphocytes SAH hydrolase is also inactivated by deoxyadenosine (188, chapter 5). Inosine in the presence of phosphate can also decrease the activity of SAH hydrolase (146). The activity is lower in erythrocytes of PNP-deficient patients (146, 190, 352).

Cyclic-AMP has also been found in elevated concentrations in erythrocytes of patients with ADA deficiency (280, 318) and in lymphocytes that were incubated with adenosine (226, 323, 415, 431). It is not clear how cyclic-AMP inhibits PHA-stimulation of lymphocytes (150, 338) and lymphocyte-mediated cytolysis (415). The role of cyclic-AMP in energy metabolism of lymphocytes needs further investigation (252).

Some important questions remain. Why do purine nucleosides prefer to accumulate in lymphoid tissues of patients, what is the origin of these nucleosides in vivo and why are they more toxic to lymphoid tissues than to other tissues? In most tissues of ADA-deficient children, ADA activity is decreased (153), but in lymphoid tissues the residual activity is the lowest. The high MW enzyme is probably responsible for the higher residual activity in liver, lung and kidney. Therefore it seems unlikely that in these tissues adenosine

and deoxyadenosine accumulate. Furthermore it was found with deoxycoformycin-treated mice that the thymus reduced in weight and accumulated more dATP than the other tissues (140, 295). The activity of SAH hydrolase was also decreased in lymphoid tissues (140, 295). ADA activity in liver, spleen, heart and thymus was equally inhibited (about 90%) by deoxycoformycin, but in intestinal tissues it was affected to a lower extent or not at all (140, 371).

The high activity of ADA in intestine will effect that adenosine and deoxyadenosine from food will be deaminated in both normal man and ADA-deficient patients. A major source for adenosine and deoxyadenosine may be the breakdown of DNA and RNA during the maturation of erythrocytes (54, 140).

Accumulation of dATP is highest in thymus of mice after injection of deoxycoformycin (140, 295). Incubation of various lymphoid cell types with deoxyguanosine showed that accumulation of dGTP is highest in thymocytes (67). It is still not clear why in other tissues this accumulation is lower. Thymic tissues of man have higher activities af deoxynucleoside kinases than other tissues (46), but in various tissues of mice adenosine kinase and deoxyadenosine kinase are present in comparable activities (49, 295).

1.9. Some aspects of purine metabolism in leukemia

There is accumulating evidence that aberrations in purine metabolism exist in distinct types of leukemia. Enzymes of purine metabolism may be markers of lymphoid malignancy (26). The first reports showed conflicting data, but now it is clear that in T-acute lymphatic leukemia the activity of ADA in lymphoblasts is increased, when compared to the activity in PBL (22, 59, 70, 88, 117, 283, 320, 335, 342). This is accompanied by an increase in TdT (59, 70). In chronic lymphatic leukemia cells the activity of ADA was lower than in PBL (88, 117, 291); the activity of PNP was also lower (215). Activity of ecto-5'-nucleotidase was significantly lower in T-acute lymphatic leukemic cells, but not in those of non-T—non-B acute lymphatic leukemia (283, 297). No significant aberrations in PNP, AK, APRT and HGPRT were found in several other kinds of leukemia (84, 232, 283, 320). A human thymus/leukemia antigen has recently been associated with a low MW form of ADA (58, 88). In addition increased amounts of ATP have been found in leukemic lymphoblasts (79), a higher activity of amido phosphoribosyltransferase in acute myeloblastic leukemia (17) and aberrant kinetics of PRPP synthetase (77, 78).

Most of the findings are in agreement with the theory of Weber (409) that in neoplastic tranformation several key enzymes have altered activities, chanelling the metabolic routes to nucleotide synthesis. Evidence was obtained from several kinds of hepatomas and tumors (182, 410). It has been argued that these key enzymes could be of importance for therapy. Especially inhibition of ADA is of interest for therapy of leukemia, since in ADA deficiency associated with SCID mainly lymphoid tissues are affected and in mice treated with deoxycoformycin, the lymphoid tissues and especially the thymus are most severely affected (140, 295). Indeed deoxycoformycin administered to patients with T-acute lymphatic leukemia, that did not respond to normal therapy, resulted in remission although the patients died of complications (236, 284, 331). Post-mortem findings indicated a complete absence of leukemic cells in all tissues. Further trials were performed and some patients kept alive (282, 312). Biochemical investigations showed an increase in plasma and urine of the concentrations of adenosine and deoxyadenosine, an inhibition of SAH hydrolase in erythrocytes, an accumulation of dATP in erythrocytes and lymphoblasts, depletion of ATP in erythrocytes, and inhibition of ADA in erythrocytes (236, 312, 331, 404). Since most other cytostatics are purine and pyrimidine analogs and mostly have to be converted to their active form by one of the purine or pyrimidine interconversion enzymes, more knowledge of purine and pyrimidine metabolism in lymphocytes could contribute to a more specific cancer chemotherapy. More details on purine metabolism in leukemia will be given in the thesis of J. van Laarhoven.

1.10. Aim and scope of this study

This investigation was started in order to get further insight into the role of ADA and PNP in immune function. Since patients with ADA or PNP deficiency have severe lymphopenia, biochemical investigation on material of these patients was mainly directed to plasma, urine, erythrocytes and fibroblasts. Patients with ADA or PNP deficiency are rare and therefore most studies have been performed on model systems that include human and other mammalian peripheral and PHA-stimulated lymphocytes, fibroblasts and a number of wild-type and mutant cell-lines of various sources. In previous comparative studies from this laboratory (Thesis Tax), it was found that equine lymphocytes have ADA activities comparable to that from patients with ADA deficiency with SCID. These studies were extended

to lymphocytes of other species (chapter 5) and it was found that porcine lymphocytes also contain very low ADA levels and that ovine and caprine lymphocytes have a very low PNP activity. Lymphocytes of these species could form model systems to study the pathophysiology of the immune dysfunction in man. Some information might be obtained by investigation of the differences in purine and pyrimidine metabolism of these "enzyme-deficient" cells. Study of the biochemical and biological effects of (deoxy)nucleosides on these cells may help to elucidate the indispensible role of ADA and PNP in human lymphocytes. Knowledge of the purine and pyrimidine metabolism of lymphocytes, lymphoblasts and thymocytes of other mammalian species may give more insight in the ontogeny and function of the lymphoid cells. This knowledge appears to be essential for development of procedures for specific immunosuppression and cancer chemotherapy. Comparative investigations were performed on purine and pyrimidine metabolism of PBL, thymocytes and splenocytes and the concentrations of several purine and pyrimidine metabolites. Furthermore the effects of mitogens, nucleosides and nucleotides were studied.

Firstly, the role of PRPP in purine and pyrimidine metabolism is described by reporting the activities of phosphoribosyltransferases (OPRT, HPRT and APRT) and PRPP synthetase and the concentration of PRPP in lymphocytes of five mammalian species (chapter 2). Chapter 3 describes the influence of PHA-stimulation on the activity of PRPP synthetase and on the concentration of PRPP in lymphocytes of man, horse and pig. Kinetics of PRPP synthetase were studied in lymphocytes of man and horse. Equine lymphocytes were used to study the effects of several metabolites and antimetabolites on the activity of PRPP synthetase. Chapter 4 deals with the effect of several of these compounds on the activity of porcine OPRT. The effect of PHA-stimulation on the activities of enzymes of de novo and salvage synthesis and degradation of pyrimidine nucleotides is studied.

In the second part of this thesis the metabolism of purine nucleosides in PBL of several mammalian species and their effects on mitogen-induced proliferation of mammalian lymphocytes is described. Chapter 5 deals with the kinetics of the enzymes that are involved in adenosine and deoxyadenosine anabolism and catabolism. In chapter 6 standardization of the conditions for PHA-stimulation is described for lymphocytes of various species. These conditions were used to study the influence of adenosine, deoxyadenosine and EHNA on thymidine, uridine and leucine incorporation (chapter 7).

Chapter 8 deals with the metabolism of guanosine, inosine and their deoxycompounds and also with their effects on mitogen-induced proliferation.

In the third part, the concentration of the purine and pyrimidine nucleotides were measured with HPLC in PBL of man, horse, pig and sheep (chapter 9). The effects of PHA-stimulation with and without adenosine or deoxyadenosine on the concentrations of ATP, TTP and dATP are also described in this chapter.

The fourth part deals with purine metabolism in cells from thymus and spleen. The possible age-dependency of ADA and PNP activities in rat lymphoid tissues is described in chapter 10 and the activities of ADA, PNP, AK, deoxyguanosine kinase and PRPP synthetase and the concentration of PRPP are reported in chapter 11.

In chapter 12 a general survey and summary of the results will be given.

Chapter 2

CONCENTRATION, SYNTHESIS AND UTILIZATION OF PRPP IN LYMPHOCYTES OF FIVE MAMMALIAN SPECIES *

2.1. Introduction

In purine and pyrimidine metabolism PRPP plays an important role. It is a substrate for HPRT and APRT, two salvage enzymes in purine metabolism, and OPRT, a key enzyme in pyrimidine de novo synthesis. The intracellular concentration of PRPP may regulate the rate of purine synthesis de novo in nucleated cells (12, 423), although this is disputed.

SCID associated with ADA or PNP deficiency might be mediated by inhibition of PRPP synthesis, or by inhibition of pyrimidine nucleotide synthesis de novo (109, 326). Since lymphocytes of horse and pig contain only a very low ADA activity and those of sheep a very low PNP activity, these cells may form models to study the pathophysiological effects of ADA and PNP deficiency (403). Information might be obtained by a comparative investigation of the purine and pyrimidine metabolism in the lymphocytes of these species. In this paper we describe our measurements of the concentration of PRPP, its synthesis and utilization by phosphoribosyltransferases in lymphocytes of these three animals and of man and cattle. Results are compared with those obtained earlier in erythrocytes (364, 366).

2.2. Materials and methods

2.2.1. Materials

Ficoll (MW 400 kD) was obtained from Pharmacia and Isopaque from Nyegaard & Co., Oslo, Norway. A Ficoll-Isopaque solution was prepared as described previously (370). {Carboxyl-^{14}C}orotic acid, {carboxyl-^{14}C}orotidine-5'-monophosphate, Omnifluor and Aquasol were obtained from the New England Nuclear Corporation, Dreieichenhain, FRG, {8-^{14}C}hypoxanthine and {8-^{14}C}adenine from the Radio-

*adapted from Peters & Veerkamp (267)

chemical Centre, Amersham, England. Plastic sheets precoated with 0.1 mm PEI-cellulose were purchased from Merck, and PRPP and a preparation from brewer's yeast, containing OPRT and ODC from Boehringer, Mannheim, FRG.

2.2.2. Animals

Blood samples were taken in heparinized bottles from healthy human volunteers and adult animals. The following species were used: man (*Homo sapiens*), Wistar rats (*Rattus norvegicus*), Texel sheep (*Ovis aries*), pig (*Sus scrofa*), horse (*Equus caballus*) and cattle (*Bos taurus*). Blood samples from pig and cattle were obtained from the local slaughterhouse. Equine and ovine blood were taken from some local farmers.

2.2.3. Preparation of lymphocyte extract

Lymphocytes were isolated by Ficoll-Isopaque gradient centrifugation (38) of blood diluted with isotonic saline. Cells were washed twice with isotonic Tris solution (50 mM Tris-HCl, pH 7.4 containing 100 mM NaCl) or PBS and counted in a hemacytometer. The cells washed with isotonic Tris solution were suspended in either 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA (for OPRT and ODC assay and determination of PRPP concentration) or in 100 mM Tris-HCl (pH 7.4) containing 25 mM $MgCl_2$ and 1 mM EDTA (for HPRT and APRT assay). The PBS-washed cells were suspended in 25 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA (for PRPP synthetase assay). Extracts of lymphocytes for all determinations were prepared by sonication (Branson sonifier, 8 bursts of 5 sec at maximal output). Lysis of lymphocytes was controlled by microscopical examination. HPRT and APRT activities were estimated in the supernatant obtained by centrifugation at 20000 g for 20 min at $4^{\circ}C$.

2.2.4. Enzyme assays

All enzyme activities were measured at $37^{\circ}C$ in a shaking water bath by radiochemical methods. Enzyme activities were expressed in nmoles of product formed per h per 10^6 cells. Linearity of the reactions in respect to time and amount of cell extract was ascertained.

The conditions for the OPRT and ODC assay in ovine lymphocytes were similar as described for those of man, cattle and horse (370).

The amount of lymphocyte extract was equivalent to $5 - 15 \times 10^6$ cells for sheep and pig. With porcine lymphocytes the substrate concentrations were higher: 0.273 mM {carboxyl-^{14}C}orotic acid (1.92 mCi/mmol) and 0.234 mM {carboxyl-^{14}C}orotidine-5'-monophosphate (38 µCi/mmol).

PRPP synthetase activity was determined by the method described previously (365). The incubation mixture (0.5 ml) contained 25 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM ATP, 1 mM ribose 5-P, 10 mM $MgCl_2$, 0.30 mM {carboxyl-^{14}C}orotic acid (2.53 mCi/mmol), 20 µg of the OPRT-ODC preparation and lymphocyte extract equivalent to $0.2 - 2 \times 10^6$ cells. $^{14}CO_2$ production was measured after 30 min incubation.

The assay for PRPP concentration in erythrocytes (365) could also be applied to lymphocytes. One-ml samples of cell extracts (equivalent to $7 - 50 \times 10^6$ cells) were kept in boiling water for 45 sec and immediately chilled on ice. After thoroughly mixing the other reagents were added. The reaction mixture (1.12 ml) contained 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 18 mM $MgCl_2$, 23 µM {carboxyl-^{14}C}orotic acid (15.6 mCi/mmol) and 0.45 mg of the OPRT-ODC preparation. After 45 min reaction was terminated by injecting 0.2 ml 5 N $HClO_4$. $^{14}CO_2$ was trapped and determined as described previously (365).

The incubation mixture (40 µl) for HPRT and APRT assays contained 50 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, 0.5 mM EDTA, 1.13 mM PRPP, lymphocyte extract equivalent to $2 - 8 \times 10^5$ cells and 0.17 mM {8-^{14}C}hypoxanthine (59 mCi/mmol) or 0.14 mM {8-^{14}C}adenine (62 mCi/mmol). After incubation for 10 - 90 min reaction was stopped by adding 10 µl 8 N formic acid. Separation of nucleotides from nucleosides and bases on PEI-cellulose and determination of radio-activity were described previously (364).

Protein content was determined according to Lowry et al. (212).

2.3. Results and discussion

PRPP concentration and PRPP synthetase activity could be measured in lymphocytes in the same way as in erythrocytes (365). The PRPP assay was linear in respect to the amount of PRPP present in the lymphocyte extract. The recovery percentage was 89 ± 4 (mean ± SD, 23 determinations). In the PRPP synthetase assay optimal concentrations of ATP, ribose 5-P and phosphate were used.

Special attention must be given to the isolation buffer of the lymphocytes. Phosphate is necessary for the conservation of PRPP synthetase activity. Isolation in the absence of phosphate results in a

Table 2.1. Concentration of PRPP and activity of PRPP synthetase in mammalian lymphocytes

Species	Protein concentration	PRPP concentration	PRPP synthetase
Man	85 ± 36 (38)	4.6 ± 2.4 (7)	5.7 ± 1.5 (12)
Cattle	45 ± 20 (23)	3.9 ± 1.4 (5)	4.4 ± 1.2 (6)
Horse	46 ± 16 (33)	5.4 ± 1.5 (8)	4.9 ± 1.2 (20)
Pig	45 ± 22 (11)	7.1 ± 2.7 (5)	5.0 ± 1.9 (7)
Sheep	41 ± 22 (6)	5.2 ± 2.3 (5)	2.6 ± 1.3 (6)
Rat	16 ± 4 (4)	N.D.	7.4 ± 3.4 (5)

PRPP concentration is given in nmol/10^9 cells, protein concentration in µg/10^6 cells and PRPP synthetase activity in nmol/hr per 10^6 cells. Values are given as the mean ± SD. Numbers in parentheses refer to the number of individuals. N.D., not determined.

rapid loss of enzyme activity which can not be restored by addition of phosphate to the incubation mixture. PRPP synthetase activity is stable for 2 - 3 hr when the cells are stored in PBS either at 20° or at 4°C. On the other hand phosphate must not be present in the isolation buffer, when the PRPP concentration has to be determined. Isolation in PBS results in five- to ten-fold higher PRPP concentrations than the use of isotonic Tris-buffer, due to PRPP synthesis. The PRPP concentration does not change when the lymphocytes are kept for 2 - 3 hr in the isotonic Tris-solution at 4°C.

Table 2.1 shows the results of our measurements of PRPP concentrations and PRPP synthetase activities. The amount of protein in the cell extracts is also given, since many literature data are based on this parameter. The PRPP concentrations and PRPP synthetase activities of lymphocytes do not show large variations between the mammalian species in contrast to those in erythrocytes (366). There is no correlation between lymphocytes and erythrocytes of the same species in respect to PRPP concentration and PRPP synthesis.

Data on PRPP concentrations and PRPP synthesis are only available on human lymphocytes, but comparison is difficult since methods of isolation of lymphocytes and parameters used for concentration and enzyme activity markedly differ. Hovi et al. (166) found a lower PRPP concentration in lymphocytes, but Allsop & Watts (6) reported comparable concentrations. Other data (74) can not be compared. PRPP synthetase activities of human lymphocytes are in the same range as values reported earlier (6, 129, 343, 426). We found a considerable

activiy of this enzyme in rat lymphocytes in contrast to Hallak & Wilkinson (129).

Activities of OPRT and ODC were estimated together, because these enzymes exist as a complex in various cell systems (114, 264, 330). Because of the instability of these enzymes, activities were measured immediately after preparation of the extract. Their activities in human, equine and bovine lymphocytes were reported earlier (370), but are presented here to compare them with the activities in the other species and with the capacity of the other two PRPP-utilizing phosphoribosyltransferases (Table 2.2).

Ovine lymphocytes show a similar OPRT activity as human and bovine lymphocytes, while their ODC activity is intermediate. Porcine lymphocytes show higher activities of OPRT and ODC than those of the other mammals. There was again no correlation between activities in lymphocytes and erythrocytes (363) of the same species. OPRT and ODC activities were to low in erythrocytes of horse and sheep (363) to conclude anything about a relationship. In the lymphocytes of these animals the same coordinate relationship could be established as in the lymphocytes and erythrocytes of the other species. Such a relationship was earlier found in human erythrocytes by Fox et al. (114) and in rat tissues (264). In porcine and human lymphocytes the ODC/OPRT ratio was markedly higher than the usually observed value of 2 - 3. A high ratio was also found in porcine erythrocytes (363).

HPRT and APRT activities were determined in the 20 000 g supernatant of lymphocyte extracts to diminish interference of membrane-bound 5'-nucleotidase. In all APRT assays adenosine accounted for less than 5% of products formed. Because of the absence of an adenosine phosphorylase no substrate dilution had to be expected. In HPRT assay only in man more than 5% of the formed products was inosine. Its formation was linear in respect to time. At the end of 30 min incubation of human lymphocyte extract the concentration of inosine could reach a value of 8 µM, which is markedly below the reported Km values of 30 - 100 µM for PNP (107, 199, chapter 8). Because no phosphate is included in the reaction mixture, its concentration will remain much below the Km value of 0.3 mM (107, 199). Furthermore incubation in the presence of excess inosine did not decrease the sum of products (IMP plus inosine). So it is not likely that a recycling via inosine to the hypoxanthine substrate of HPRT takes place. Because of the heat stability of HPRT and APRT in lymphocyte extracts, reactions were not terminated with heating as on hemolysates (363) but with formic acid. Since data on purine phosphoribo-

Table 2.2. Activities of HPRT, APRT, OPRT and ODC and the ODC/OPRT ratio in extracts of mammalian lymphocytes

Species	HPRT	APRT	OPRT	ODC	ODC/OPRT
Man	6.0 ± 1.6 (9)	4.8 ± 2.2 (7)	0.39 ± 0.19 (11)	1.37 ± 0.44 (9)	4.8 ± 2.7 (9)
Cattle	2.0 ± 0.7 (7)	0.3 ± 0.3 (5)	0.47 ± 0.16 (9)	0.68 ± 0.19 (9)	1.5 ± 0.3 (9)
Horse	23.2 ± 4.6 (6)	4.5 ± 1.4 (4)	0.06 ± 0.01 (5)	0.16 ± 0.03 (5)	2.7 ± 0.6 (5)
Pig	5.8 ± 1.9 (7)	0.4 ± 0.1 (5)	1.15 ± 0.49 (9)	5.79 ± 2.17 (8)	6.5 ± 2.1 (6)
Sheep	4.8 ± 1.2 (7)	0.4 ± 0.3 (4)	0.34 ± 0.14 (6)	0.90 ± 0.24 (6)	2.7 ± 0.4 (6)

Enzyme activities are given as the mean ± SD in nmol/hr per 10^6 cells. The number of individuals is given within parentheses.

syl transferases of human lymphocytes are sometimes based on the protein content of the 20 000 g supernatant we also measured this parameter. The content was about 20 µg protein per 10^6 cells.

In man activities of HPRT and APRT were about the same and comparable to those in the literature (74, 84, 287, 320). Most authors found a somewhat higher activity of APRT. The large variations in the actvities of these enzymes and also of other enzymes (366) in lymphocytes of pig and cattle could be caused by the heterogenous population of these animals, since blood was obtained at the local slaughterhouse. In each individual animal HPRT activity was always higher than APRT actvity. APRT actvity was very low in lymphocytes of cattle, pig and sheep, but a higher and similar activity was present in lymphocytes of horse and man. The highest HPRT activity was found in equine lymphocytes, while this enzyme was nearly absent from erythrocytes of this animal (363). In man both types of blood cells contain a considerable activity of HPRT and are affected in complete or partial deficiency of this enzyme (74).

In mammalian lymphocytes no direct correlation was found between the maximal activities of OPRT, HPRT and APRT, and between these actvities and the PRPP concentration or activity of PRPP synthetase. In lymphocytes of all species except cattle total activities of these phosphoribosyl transferases were higher than that of PRPP synthetase. HPRT seems to be the most important PRPP consumer out of the enzymes tested. In all species OPRT activity is much lower. Since adenine, the substrate of APRT, is nearly absent from mammalian cells, the high APRT activity in human and equine lympho-

cytes is not clear. Maximal HPRT activity of human lymphocytes is about equal to the capacity of PRPP synthetase. The same holds for PRPP amidotransferase (6). The Km values of HGPRT and APRT for PRPP however are about an order of magnitude lower than that of the amidotransferase (418). Thus the salvage pathways will have a marked competitive advantage for the low concentration of PRPP in PBL. Mitogenic stimulation activates both de novo and salvage synthesis of purine nucleotides (168, 287). The role of PRPP in this respect, however, is still discutable (6, 74, 141, 168) and deserves further investigation.

2.4. Summary

1. The concentration of PRPP and the activities of PRPP synthetase, OPRT, ODC, HPRT and APRT have been determined in lymphocyte extracts of man, cattle, horse, pig and sheep. 2. PRPP concentration and PRPP synthetase actvity were similar in all species. OPRT and ODC activities were very low in equine lymphocytes and relatively high in porcine lymphocytes. 4. OPRT and ODC activities were coordinate in all species studied. 5. HPRT activity was higher in equine lymphocytes than in those of the other species. HPRT activity was higher than APRT activity in all animal species studied. 6. APRT activity was similar in equine and human lymphocytes and very low in those of sheep, cattle and pig.

Chapter 3

METABOLISM OF PRPP IN PERIPHERAL AND PHA-STIMULATED MAMMALIAN LYMPHOCYTES*

3.1. Introduction

Intracellular availability of purine, pyrimidine and pyridine nucleotides in adequate concentrations is necessary for the survival of every animal cell. PRPP plays a key role in the synthesis of these nucleotides not only as a substrate (Fig. 3.1) but also as a regulator of several enzymes e.g. in pyrimidine biosynthesis of CPS II (359). Hence the regulation of PRPP synthesis is important for the overall control of nucleotide metabolism.

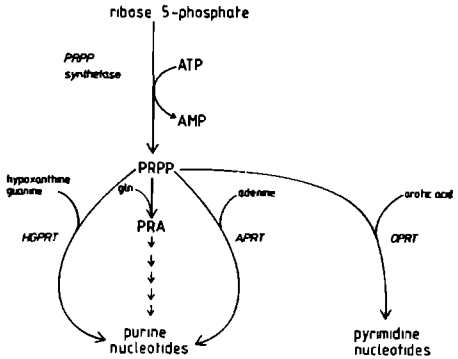


Fig. 3.1. Metabolism of PRPP.
PRA, phosphoribosylamine; and gln, glutamine.

PRPP synthesis from ATP and ribose 5-P (Fig. 3.1) is catalyzed by PRPP synthetase. The activity of this enzyme isolated from human erythrocytes (105), bacteria (355), Ehrlich ascites cells (417) and rat liver (306) appeared to be influenced by several nucleotides. PRPP synthesis and regulation of PRPP synthetase activity is affected in certain cases of gout (21) and ADA deficiency associated with SCID might be mediated by inhibition of PRPP synthesis (118).

Lymphocytes stimulated with the mitogen PHA are an in vitro system in which their transformation to lymphoblasts can be studied. This process is associated with enhanced RNA and DNA synthesis and

*adapted from Peters et al. (270)

an increase of several enzyme activities (211). The role of PRPP synthetase and the intracellular concentration of PRPP during this process are disputed (6, 74, 166, 343).

Previously we reported studies on the metabolism of PRPP in PBL of various mammals (chapter 2). We extended our studies to the concentration of PRPP and the activity of PRPP synthetase in PHA-stimulated lymphocytes of man, horse and pig. Equine and porcine lymphocytes contain a very low ADA activity and may serve as model systems in studying the effects of ADA deficiency on lymphocyte function (403). We also studied the kinetics of PRPP synthetase and tested 35 compounds of interest including 14 adenosine derivatives on their influence on its activity.

3.2. Materials and methods

3.2.1. Materials

Origin of most materials is described in chapter 2. {6-^{3}H}Thymidine was obtained from the Radiochemical Centre, Amersham, UK. Bacto Phytohemagglutinin-P was a product from Difco Laboratories, Detroit, MI, USA. Autopow Minimum Essential Medium Eagle for suspension cultures and horse serum were from Flow Laboratories, Irvine, Scotland, UK; Soluene-100 from Packard, Groningen, the Netherlands. All other chemicals were from the highest quality commercially available.

Blood samples were taken in heparinized bottles from healthy adult volunteers and adult horses (Equus caballus) and pigs (Sus scrofa)

3.2.2. Enzyme assays

Preparation of lymphocyte lysates, determination of the activity of PRPP synthetase and of the concentration of PRPP were performed as described in chapter 2. In the assay of PRPP synthetase the concentrations of potassium phosphate and $MgCl_2$ were 25 and 10 mM, respectively. ATP and ribose 5-P were present in a 1 mM concentration, unless otherwise indicated. Interpretation of kinetics and calculation of Ki values were performed according to Segel (328).

3.2.3. Cell cultures

Cells were cultured in a volume of one ml in silanized glass

tubes in 22 mM bicarbonate-buffered MEMS supplemented with horse serum (10% for human and porcine lymphocytes and 20% for equine lymphocytes), 2 mM L-glutamine, and 100 µg streptomycin and 100 Units penicillin per ml under an atmosphere of 95% air/5% CO_2 at 37°C and 90-95% humidity. Cell concentration was 2×10^6 cells/ml for equine and porcine lymphocytes and $0.5 - 1.0 \times 10^6$ cells/ml for human lymphocytes. In PHA-stimulated cultures the concentration of PHA was 20, 5 and 1.25 µg PHA/10^6 cells for human, porcine and equine lymphocytes, respectively.

Cells were isolated in PBS for use in cultures in which the activity of PRPP synthetase was measured. At 24, 48 and 72 hr after starting the cultures, the contents of 2 tubes were pooled, washed with PBS, centrifuged and suspended in an appropriate amount of 25 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. Cells were lysed by sonication and the activity of PRPP synthetase was measured. Concentration of PRPP was estimated in cells isolated in isotonic Tris buffer (chapter 2). After 3, 24, 48 and 72 hr the contents of 7-10 tubes were pooled, washed with isotonic Tris-buffer, centrifuged and suspended in an appropriate amount of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. Cells were lysed and the concentration of PRPP was determined.

At 24, 48 and 72 hr thymidine incorporation was determined after adding 18 µM [6-^{3}H]thymidine (1 Ci/mmol) to the culture 4 hr prior to harvest. The cells were isolated with a Titertek Cell Harvester (Flow Laboratories, Irvine, UK) using TCA precipitation. The glass-fiber filters were dried and 0.2 ml of Soluene-100 and 5 ml of toluene, containing 6 g PPO and 0.4 g dimethyl-POPOP per l, were added. Radioactivity was measured in a Packard PRIAS Tri-Carb Liquid Scintillation Counter.

3.3. Results

3.3.1. Effects of PHA-stimulation

The culture conditions of lymphocytes, optimized for [6-^{3}H]thymidine incorporation (chapter 6), were also used to follow activity of PRPP synthetase and the concentration of PRPP. In PHA-stimulated cultures thymidine incorporation reaches a optimum at 2 or 3 days. Stimulation index (ratio of thymidine incorporated into PHA-stimulated cultures to that into control cultures) at 48 and 72 hr was always higher than 8.

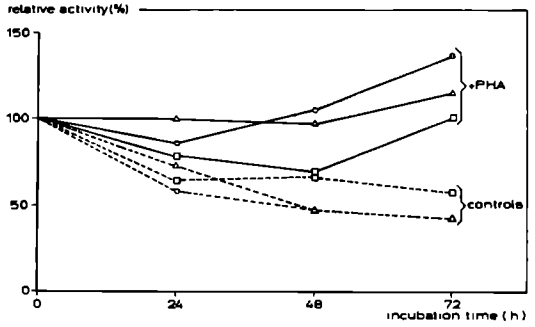


Fig. 3.2. Effect of PHA-stimulation on the activity of PRPP synthetase. Values (in % of the activity at the start of the cultures) are means of 4 experiments. SD was always less than 20% of the mean. Activity of PRPP synthetase at 0 hr was 5.2 ± 0.7, 7.1 ± 2.1 and 6.4 ± 1.8 nmol/hr per 10^6 cells for equine (o), human (□) and porcine (Δ) lymphocytes, respectively (means ± SD).

Fig. 3.2 gives the relative activity of PRPP synthetase of control and stimulated lymphocytes. In non-stimulated cells PRPP synthetase activity markedly decreased at cultivation, presumably by death and degradation of the cells. In equine lymphocytes the activity of PRPP synthetase is slightly increased with stimulation, in the other species no significant change was found. In contrast concentration of PRPP rapidly increased in cultures with and without PHA (Fig. 3.3). In cultures without PHA concentration of PRPP falls down to control levels at 24 hr. In human and porcine lymphocytes the increase of PRPP concentration at 3 hr is larger in cultures with PHA than in cultures without PHA. PRPP concentration reaches a maximum at 48 hr

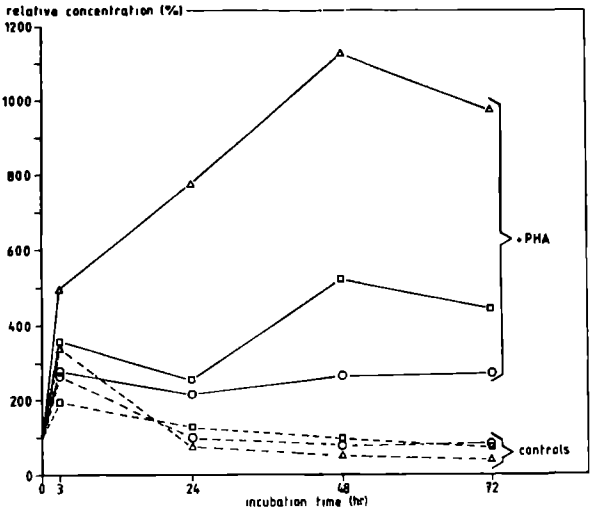


Fig. 3.3. Effect of PHA-stimulation on the concentration of PRPP. Values (in % of the concentration at the start of the culture) are means of 3-9 experiments. SD was always lower than 30% of the mean. Concentration of PRPP at 0 hr was 4.7 ± 2.5 (9), 7.7 ± 3.0 (7) and 8.1 ± 3.0 (6) nmol/10^9 cells for equine (o), human (□) and porcine (Δ) lymphocytes, respectively (means ± SD for the number of experiments given within parentheses).

in porcine and human lymphocytes, while in equine lymphocytes the values at 48 and 72 hr are comparable.

3.3.2. Kinetics of PRPP synthetase

Km values of ATP and ribose 5-P were determined from Lineweaver-Burke plots at the optimal concentrations of the second substrate (1 mM). PRPP synthetase showed similar Km values for ribose 5-P and comparable biphasic kinetics for ATP in lysates from lymphocytes of man and horse (Table 3.1). With all ribose 5-P concentrations until 1 mM no substrate inhibition by ATP was observed until 1 mM ATP. Substrate inhibition by ribose 5-P was dependent on the ratio of ribose 5-P/ATP and was observed at ratios higher than 2 (Fig. 3.4). At standard assay conditions (1 mM ATP, 1 mM ribose 5-P) maximal activities were observed.

Table 3.1. Km values for ATP and ribose 5-P of PRPP synthetase in lysates of human and equine lymphocytes.

	Man	Horse
ATP	4.7 ± 1.1 (3)	6.1 ± 0.9 (12)
	21.5 ± 5.3 (4)	38.5 ± 4.0 (5)
Ribose 5-P	21.2 ± 5.1 (7)	27.5 ± 2.1 (4)

Values (in µM) are means ± SD for the number of experiments given within parentheses.

3.3.3. Inhibition of PRPP synthetase

Because of comparable kinetics and larger availability than human lymphocytes we used equine lymphocytes to study the effects of several purines, pyrimidines and some other compounds of interest on the activity of PRPP synthetase both at suboptimal ATP and suboptimal ribose 5-P concentrations (Table 3.2). Prior to testing the effect on PRPP synthetase, their non-interference with the added OPRT and ODC enzymes from yeast was ascertained at suboptimal concentrations of PRPP (8 µM). None of the tested compounds was inhibitory, even TTP, a strong inhibitor of hemolysate OPRT (364) and AMP and CMP, inhibitors of hemolysate ODC (369), did not inhibit.

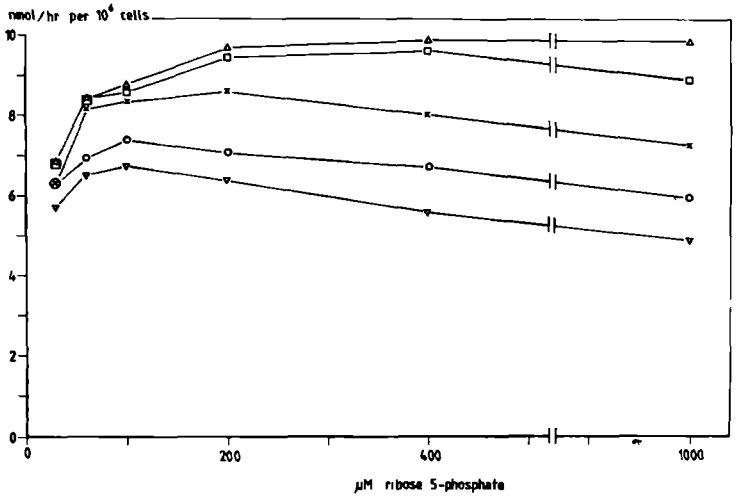


Fig. 3.4. Effect of substrate concentrations on PRPP synthetase activity of human lymphocytes. ∇, 30.3 μM ATP; o, 58.8 μM ATP; X, 150 μM ATP; □, 500 μM ATP; and Δ, 1000 μM ATP

PRPP synthetase activity was inhibited by nearly all nucleotides tested in a 5 mM concentration, either with respect to ATP or ribose 5-P (Table 3.2). Cyclic GMP and nucleosides did not show any inhibitory effect. The monophosphate nucleotides AMP, dAMP, GMP and dGMP and the di- and triphosphate nucleotides ADP, dADP, GDP, dATP and GTP inhibited strongly, the monophosphates more pronounced at low ATP and the triphosphates at low ribose 5-P concentrations. Deoxycompounds were less inhibitory than their ribonucleotides. ATP showed substrate inhibition at this high concentration. Inhibition with pyrimidine nucleotides was less than with purine nucleotides. Of the other compounds tested only SAM inhibited the enzyme markedly.

The strongest inhibitors and cyclic-GMP were also tested at 0.1 mM concentration (Table 3.3) to get more insight in the kind of inhibition. Only the adenine and guanine nucleotides were inhibitory at suboptimal ATP concentration, but not at suboptimal ribose 5-P concentration. Therefore we determined Ki values for AMP, dAMP, ADP and dADP at a range of low ATP concentrations (5-30 μM). Values are given in Table 3.4. Inhibition by AMP is competitive as is shown in a Lineweaver-Burke plot (Fig. 3.5). At high concentrations of ATP inhibition changes to non-competitive and a higher Ki value is found. With the other nucleotides different kinds of kinetics were observed,

Table 3.2. Effects of several purines and pyrimidines and some other compounds of interest on the activity of PRPP synthetase from equine lymphocytes.

Addition	Relative activity at	
	1 mM ribose 5-P 30 µM ATP	30 µM ribose 5-P 1 mM ATP
None	100	100
Adenosine	82 ± 6	100 ± 2
Deoxyadenosine	86 ± 11	94 ± 10
Cyclic-AMP	75 ± 8	56 ± 1
AMP	2.6 ± 1.4	3.9 ± 1.9
dAMP	6.4 ± 1.2	14 ± 2
ADP	1.3 ± 0.1	0.7 ± 0.3
dADP	23 ± 5	20 ± 5
ATP	14 ± 1	9.4 ± 3.5
dATP	44 ± 9	6.1 ± 3.1
Cordycepin*	97 ± 4	104 ± 1.7
Cordycepin-monophosphate*	4.9 ± 1.2	5.6 ± 1.0
Cordycepin-triphosphate*	87 ± 10	68 ± 7
Guanosine	95 ± 10	99 ± 5
Deoxyguanosine	92 ± 5	96 ± 5
Cyclic-GMP	87 ± 11	96 ± 1
GMP	16 ± 2	27 ± 1
dGMP	29 ± 2	54 ± 4
GDP	4.0 ± 0.8	8.3 ± 2.9
GTP	5.5 ± 0.7	4.9 ± 0.3
dGTP	62 ± 6	59 ± 4
Inosine	93 ± 10	105 ± 2
Deoxyinosine	103 ± 6	107 ± 5
IMP	22 ± 2	61 ± 4
dIMP	92 ± 6	97 ± 9
CMP	50 ± 2	73 ± 5
CTP	37 ± 5	20 ± 2
TMP	79 ± 5	100 ± 9
TTP	103 ± 15	59 ± 15
UMP	37 ± 2	68 ± 3
UTP	111 ± 7	47 ± 6
dUTP	139 ± 10	100 ± 15
2,3-Diphosphoglycerate	95 ± 12	103 ± 11
Homocysteine	101 ± 10	102 ± 8
SAH	93 ± 8	102 ± 1
SAM	2.3 ± 1.3	2.8 ± 1.3

Values (in % of the control activity) are the means ± SD of 3-4 experiments with additions in a 5 mM (*1 mM) final concentration. Activities of PRPP synthetase (in nmol/hr per 10^6 cells) at 1 mM ATP and 1 mM ribose 5-P, at 30 µM ATP and 1 mM ribose 5-P, and at 1 mM ATP and 30 µM ribose 5-P were 5.3 ± 1.5 (23). 2.9 ± 0.7 (22) and 3.5 ± 0.7 (19), respectively (means ± SD for the number of experiments given within parentheses).

Table 3.3. Effects of several purines at 0.1 mM concentration on the activity of PRPP synthetase from equine lymphocytes

Addition	Relative activity at	
	1 mM ribose 5-P 30 µM ATP	30 µM ribose 5-P 1 mM ATP
None	100	100
AMP	16 ± 3	85 ± 6
dAMP	42 ± 7	85 ± 12
ADP	54 ± 6	93 ± 7
dADP	60 ± 6	100 ± 8
Cyclic-GMP	97 ± 3	100 ± 5
GMP	44 ± 8	90 ± 8
dGMP	87 ± 2	97 ± 9
GTP	122 ± 8	94 ± 6
IMP	84 ± 6	95 ± 8
SAM	99 ± 3	100 ± 5

Values (in % of the control activity) are means ± SD of 3-4 experiments with additions in a 0.1 mM final concentration.

by testing them at 25 and 50 µM concentration and at several different ATP concentrations. Ribose 5-P concentration was 1 mM. AMP showed the lowest Ki value. Ki values of deoxynucleotides were higher than those found for their ribonucleotides. This agrees with their percentage of inhibition (Table 3.2 and 3.3). dAMP showed non-competitive inhibition and ADP uncompetitive. The mode of inhibition by dADP was linear mixed-type.

3.4. Discussion

PHA-stimulation of lymphocytes induces a number of changes in cell (211). We confirmed the results of several authors who reported an increase in PRPP concentration in human lymphocytes in the early phase after PHA-addition (74, 166, 343, 419). Allsop & Watts (6), however, did not find an increase in PRPP concentration neither in the early phase nor after 72 hr. No reports are available on the PRPP concentration in PHA-stimulated lymphocytes of horse and pig. In all mammals the increase of PRPP concentration is not a result of a higher activity of PRPP synthetase. No alteration in the activity of PRPP synthetase of human lymphocytes after PHA-stimulation was also found by Snyder et al. (343), Allsop & Watts (6) and Danks & Scholar (77). Last authors, however, found some deviation from normal values

Table 3.4. Ki values of AMP, dAMP, ADP and dADP with respect to ATP for PRPP synthetase of equine lymphocytes.

Compound	Ki value
AMP	7.0 ± 1.7 (5)
dAMP	41.7 ± 8.1 (5)
ADP	59.1 ± 13.0 (4)
dADP	51.7 ± 11.5 (4)

Values (in µM) are means ± SD for the number of experiments given within parentheses.

in substrate inhibition constants.

Biphasic kinetics of PRPP synthetase were not previously observed, probably because most authors did not use low ATP concentrations for their Km determination. Our high Km values for ATP in human and equine lymphocytes are comparable to those found in human and leukemic lymphocytes (77), erythrocytes (20, 105), Ehrlich ascites cells (417), bacteria (354) and rat hepatoma cells (126). The Km values for ribose 5-P reported by these authors are much higher than our values. Only in human erythrocytes comparable values were found by Fox & Kelley (105) and Yip & Balis (427).

Substrate inhibition by ATP and ribose 5-P was also observed for human lymphocytes by Danks & Scholar (77), but at a lower concentration than we did, probably because they used a purified enzyme preparation. Furthermore substrate inhibition by ribose 5-P seems to depend on the concentration of ATP. At high ATP concentrations a much higher concentration of ribose 5-P is needed for substrate inhibition as was also found for rat liver by Roth & Deuel (306).

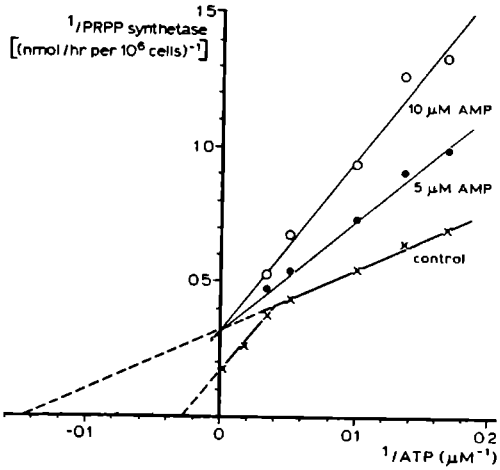


Fig. 3.5. Inhibition of PRPP synthetase in lysate of equine lymphocytes in the presence of 1 mM ribose 5-P.

Inhibition of PRPP synthetase by AMP and ADP is a common feature for the enzyme of various sources e.g. human erythrocytes (105), rat liver (306), Ehrlich ascites cells (417), spinach (10) and bacteria (355). The last authors also observed a considerable inhibition by dAMP and dADP. In all cases reported (105, 306, 355, 417) guanine and pyrimidine nucleotides were less inhibitory than adenine nucleotides. Inhibition of activity by nucleosides was never found but they can inhibit PRPP synthesis in intact cells by interfering in ribose 5-P synthesis (171) by altering the Pi content (278) or by conversion to nucleotides. The inhibition by cordycepin observed by Tyrsted & Sartorelli (383) is possibly due to the last mechanism since cordycepin-monophosphate is a potent inhibitor PRPP synthetase (Table 3.2). Unlike Garcia et al. (118) we did not observe stimulation of PRPP synthetase by IMP and GMP, but an inhibition like most authors. Cyclic-GMP had no effect at 0.1 and 5 mM concentration, although this compound was reported to be stimulatory in mammalian lymphocytes (118).

The inhibition of the enzyme by SAM was not previously observed and is remarkable because the related compound SAH did not show any inhibition. Inhibition by dATP can be considered as substrate inhibition since it can replace ATP as a pyrophosphoryl donor (307). 2,3-Diphosphoglycerate, considered as a potent regulator of PRPP synthetase in erythrocytes (423, 427), may not play this role in lymphocytes, because even at 5 mM no inhibition was observed. Stabilization of the enzyme by EDTA and a high amount of protein may cause this difference in inhibition (306). Most Ki values of AMP, ADP and dADP were estimated at high concentrations (>25 µM) of ATP and vary considerably between the enzyme preparations of various origins. Therefore no proper comparison with our results is possible. Only the value and kinetics observed for AMP in rat hepatoma cells by Green & Martin (126) are comparable.

The observed kinetics of PRPP synthetase could possibly explain the increase of PRPP concentration found after PHA-stimulation and the possible role of PRPP in ADA deficiency associated with SCID. An enlarged PRPP availablity may stimulate nucleotide and therefore RNA and DNA synthesis. Because the maximal activity of PRPP synthetase is not increased in PHA-stimulated lymphocytes, PRPP synthesis may be stimulated by an increased availability of substrates or less inhibition by regulatory inhibitors. Probably a lower concentration of AMP and ADP is present in PHA-stimulated lymphocytes together with a higher energy charge. These conditions may stimulate PRPP synthesis (11, 355). Ribose 5-P synthesis could be stimulated by an

acceleration of the hexose monophosphate shunt (134, 231).

In patients with ADA-deficiency associated with SCID an accumulation of (deoxy)adenosine will occur (206) which may lead to a higher concentration of (d)AMP. The concentration of inosine may be lower and less ribose 1-P will be formed by phosphorylysis. Furthermore accumulation of SAM can occur (281). All these processes can inhibit PRPP synthesis, which affects purine, pyrimidine and pyridine synthesis and subsequently RNA and DNA synthesis, resulting in a severe impairment of cell proliferation. Further investigations on the role of adenosine and deoxyadenosine in PRPP synthesis can give more insight in this complex metabolism.

3.5. Summary

1. PHA-stimulation increased 3-11 fold the concentration of PRPP in lymphocytes of man, horse and pig, but did not change the activity of PRPP synthetase in these cells. 2. PRPP synthetase showed similar Km values for ribose 5-P (about 25 µM) and comparable biphasic kinetics for ATP in lysates from lymphocytes of man and horse (low Km value is about 5 µM). 3. At all concentrations of ribose 5-P (30-1000 µM) no substrate inhibition by ATP was found until 1 mM. Substrate inhibition by ribose 5-P was observed at ribose 5-P/ATP ratios higher than 2. 4. The influence of 35 compounds was tested on the activity of PRPP synthetase from equine lymphocytes. Adenine and guanine nucleotides inhibited markedly more than pyrimidine nucleotides at 5 mM concentration. Inhibition by monophosphate nucleotides was more pronounced at 30 µM ATP than at 30 µM ribose 5-P. 5. At 0.1 mM only AMP, dAMP, ADP, dADP and GMP were inhibitory at 30 µM ATP, but not at 30 µM ribose 5-P. AMP showed a competitive inhibition with ATP and a Ki value of about 7 µM. dAMP, ADP and dADP showed higher Ki values with different types of inhibition.

Chapter 4

PYRIMIDINE METABOLISM IN PERIPHERAL AND PHA-STIMULATED MAMMALIAN LYMPHOCYTES*

4.1. Introduction

Pyrimidine nucleotides can be synthesized via a de novo pathway and via a salvage pathway. The contribution of both pathways varies among the various cell types and organs and conditions (131, 208, 285, 308). In human lymphocytes both pathways are present (178, 180, 213, 214, 370). At PHA-stimulation the activities of the enzymes involved in salvage pathways show a higher increase than those of the de novo pathway (180, 213, 214, 243).

Large differences exist in pyrimidine metabolism of lymphocytes among various mammalian species. The activities of OPRT and ODC are very low in lymphocytes of horse, but relatively high in those of pig (chapter 2). However, both species have a very low activity of ADA in their lymphocytes (chapter 5). A comparable activity of this enzyme in human lymphocytes is associated with SCID (122, 261). The effects of ADA deficiency could be mediated by inhibition of pyrimidine nucleotide synthesis (125, 177). Adenosine and deoxyadenosine inhibited thymidine and uridine incorporation of PHA-stimulated lymphocytes of horse and man but with porcine lymphocytes a stimulation was found (chapter 7). Therefore we performed a study on pyrimidine metabolism of their peripheral and PHA-stimulated lymphocytes. For comparison we also measured the activities of uridine kinase and uridine phosphorylase in peripheral ovine lymphocytes, since these lymphocytes have a very low PNP activity (chapter 5) that approximates the activity found in patients with PNP deficiency associated with severe T-cell dysfunction.

Although it has been argued that pyrimidine nucleotide synthesis could be inhibited in patients with ADA or PNP deficiency, this possibility has only been studied in murine cell lines (177) and erythrocytes (110, 112). Therefore we studied the effects of several metabolites that can accumulate in ADA and PNP deficiency on the activities of OPRT and ODC of porcine lymphocytes.

*adapted from Peters et al. (275)

4.2. Materials and methods

4.2.1. Materials

Origin of most materials was previously described (370, chapter 3). {6-^{3}H}Thymidine and {5-^{3}H}uridine were obtained from the Radiochemical Centre, Amersham, UK. Blood samples were taken in heparinized bottles from healthy adult volunteers and adult horses (Equus caballus), pigs (Sus scrofa), Texel sheep (Ovis aries) and cattle (Bos taurus).

4.2.2. Methods

Preparation of lymphocyte lysates and extracts, and determination of the activities of OPRT, ODC, uridine kinase and uridine phosphorylase were previously described (370, chapter 2). For the determination of the influence of several metabolites a suboptimal PRPP concentration (16 µM) was used for the OPRT reaction and a suboptimal concentration of 94 µM {carboxyl-^{14}C}-orotidine 5'-monophosphate (2.6 mCi/mmol) for the ODC reaction. Reaction time was 60 min for the OPRT and 15 min for the ODC reaction.

Cells were cultured in the presence and absence of PHA in a volume of 1 ml as described in chapter 3. After 24, 48 and 72 hr the contents of 7-10 tubes were pooled, washed with isotonic Tris buffer (pH 7.4) and suspended in an appropriate volume of 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA. Cells were lysed by sonication (8 bursts of 5 sec at maximal output, Branson sonifier) and the activities of OPRT, ODC, uridine kinase and uridine phosphorylase were determined.

Measurement of thymidine and uridine incorporation is described in chapter 3. Stimulation index (ratio of thymidine or uridine incorporation in PHA-stimulated cultures to thymidine or uridine incorporation in cultures without PHA) was at least 4 for thymidine and uridine incorporation of equine lymphocytes and at least 10 for thymidine and uridine incorporation of porcine lymphocytes, when measured at 48 hr after starting the culture. Only cultures were used for enzyme assays that showed an appropriate stimulation of thymidine and/or uridine incorporation.

4.3. Results

4.3.1. Enzyme acivities in PBL

Table 4.1. Activities of uridine kinase and uridine phosphorylase in extracts of mammalian lymphocytes

Species	Uridine kinase	Uridine phosphorylase
Man	0.53 ± 0.15 (8)	2.25 ± 0.36 (6)
Horse	0.40 ± 0.19 (11)	0.68 ± 0.49 (8)
Pig	0.040 ± 0.034 (7)	4.5 ± 3.5 (11)
Sheep	0.022 ± 0.011 (3)	1.48 ± 0.69 (4)
Cattle	0.48 ± 0.35 (8)	1.90 ± 0.91 (4)

Enzyme activities (in nmol/hr per 10^6 cells) are means ± SD for the number of individuals given in parentheses. Enzyme activities were measured in 7000 g supernatants.

All enzyme assays were proportional with time, substrate and the amount of lysate or extract. Table 4.1 gives the activities of uridine kinase and uridine phosphorylase in peripheral lymphocytes of man, horse, pig, sheep and cattle. The activities of human, equine and bovine lymphocytes were previously reported (370), but are included for comparison and since more values were obtained. Activity of uridine kinase is low in ovine lymphocytes. Activity of uridine kinase in porcine lymphocytes varied considerably. With lymphocytes of most pigs the low activity shown in Table 4.1 was found, but there were also pigs that had a significantly higher activity (1.4 ± 0.3 nmol/hr per 10^6 cells, mean ± SD of 5 individuals). The characteristics of the enzyme of the two subpopulations were not further investigated. The activity of uridine phosphorylase is higher than that of uridine kinase in lymphocytes of all species.

4.3.2. Effect of PHA-stimulation

PHA-stimulation of equine lymphocytes results in an increase in the activity of uridine kinase (Fig. 4.1). In most experiments with equine lymphocytes maximal stimulation of uridine kinase activity was found at 48 hr, at which time uridine incorporation also reached a maximum. With porcine lymphocytes the degree of the increase of uridine kinase at PHA-stimulation varied considerably with each animal. In Fig. 4.1 the results of cultures with a low and a high activity at the start of the culture are shown. Cultures that

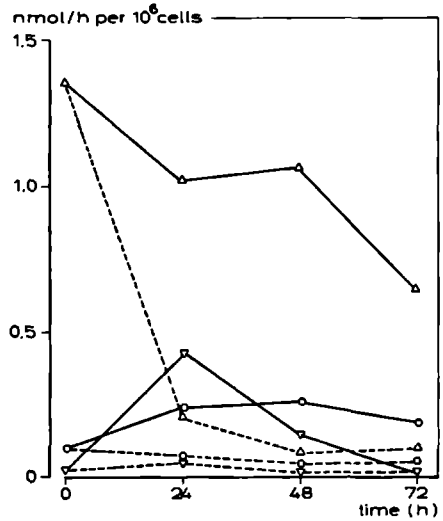


Fig. 4.1. Effect of PHA-stimulation on the activity of uridine kinase in lymphocytes of horse and pig. Solid lines represent activity in the presence of PHA and broken lines in the absence of PHA. Values of one representative experiment are shown for horse (o) out of 6, for pig with a low activity at the start of the culture (▽) one out of 4, and for pig with a high activity at the start of the culture (Δ) one out of 3.

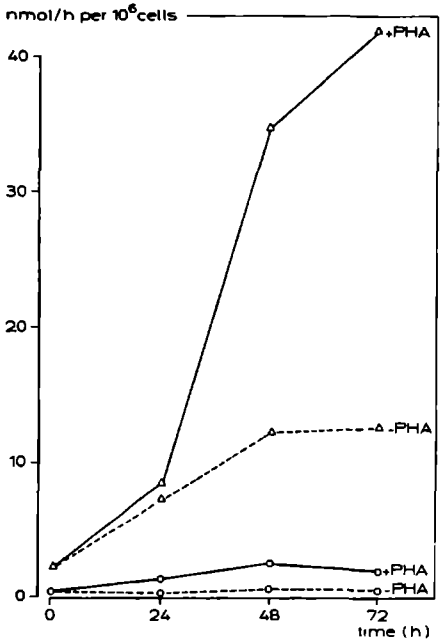


Fig. 4.2. Effect of PHA-stimulation on the activity of uridine phosphorylase in lymphocytes of horse (o) and pig (Δ). Values are those of one representative experiment out of 7 for both species.

had a low activity of uridine kinase at the start, showed a marked increase at PHA-stimulation. When a high activity was present at the start of the culture a decrease in the activity of uridine kinase was found, although the uridine incorporation in PHA-stimulated cultures was higher at 48 than at 24 hr and was at least 10 times higher than in cultures without PHA. The activity remained higher or comparable to that of porcine PHA-stimulated lymphocytes with a low activity at the start of the culture. Without PHA the decrease in activity of uridine kinase was always higher than with PHA.

Activity of uridine phosphorylase markedly increased in all cultures of equine and porcine lymphocytes at PHA-stimulation (Fig. 4.2). With porcine lymphocytes the increase at 48 hr ranged from 2-15 times the value at the start of the culture and with equine from 2-6. The variations are probably related to the various stages of the cell cycle in which the cells are taken (392). In porcine lymphocytes the activity of uridine phosphorylase also increased in cultures without PHA.

Since porcine lymphocytes showed relatively high activities of OPRT and ODC (chapter 2) we used these cells to follow the activities of OPRT and ODC during PHA-stimulation (Table 4.2). A decrease in both activities was observed in cultures with and without PHA. The higher decrease of OPRT activity is probably due to the higher instability of OPRT than ODC (364).

Table 4.2. Effects of PHA-stimulation on the activities of OPRT and ODC of porcine lymphocytes

Enzyme	PHA addition	Relative activity (in % of t=0)		
		24 hr	48 hr	72 hr
OPRT	-	39 ± 10	37 ± 34	16 ± 19
	+	54 ± 37	30 ± 20	41 ± 47
ODC	-	61 ± 20	40 ± 22	40 ± 16
	+	72 ± 18	59 ± 16	55 ± 21

Values are means ± SD of 6 experiments. Activities of OPRT and ODC at the start of the culture were 1.36 ± 0.62 and 7.24 ± 0.81 nmol/hr per 10^6 cells, respectively (means ± SD).

4.3.3. Inhibition of OPRT and ODC

We also used porcine lymphocytes to measure the effects of several purines and pyrimidines on lymphocyte OPRT activity at suboptimal PRPP concentration (Table 4.3). Since measurement of OPRT is based on the subsequent decarboxylation catalyzed by ODC, we first tested the effect of these compounds on this enzyme at a concentration of OMP that is suboptimal for the assay in porcine lymphocytes (chapter 2). Tax & Veerkamp (369) showed with human hemolysate that the decarboxylation assay of OPRT can be used to measure the effects on OPRT with compounds that inhibit the ODC. Only CMP and UMP inhibited ODC activity from porcine lymphocytes by about 25 and 55%, respectively. The inhibition of OPRT by these compounds is at least partially due to inhibition of ODC. The other compounds did not inhibit the ODC assay and $^{14}CO_2$ production was not increased by a pre-

Table 4.3. Effect of several purines and pyrimidines on the OPRT activity of porcine lymphocytes

Compound	Relative activity	Compound	Relative activity
Guanosine	66 ± 2	Adenosine	55 ± 6
Deoxyguanosine	49 ± 4	Deoxyadenosine	73 ± 6
GMP	50 ± 8	AMP	67 ± 8
dGMP	87 ± 20	Cyclic-AMP	99 ± 6
GTP	59 ± 9	dAMP	76 ± 5
dGTP	38 ± 10	ADP	78 ± 8
		ATP	85 ± 7
UMP	28 ± 7	dATP	86 ± 3
UTP	57 ± 11	SAH	76 ± 4
CMP	48 ± 7	SAM	77 ± 4
CTP	53 ± 7		
TMP	84 ± 7		
TTP	60 ± 16		

Values (in % of the control activity) are means ± SD of 3-5 experiments. Tested compounds were added in a final concentration of 5 mM. Activity of OPRT at suboptimal PRPP concentration (16 µM) was 1.24 ± 0.24 nmol/hr 10^6 cells (mean ± SD of 8 experiments)

longation of the second incubation period of the OPRT assay after injection of EDTA, that only stops the OPRT reaction by complexing the Mg^{++} ions but does not affect ODC activity. Therefore the data in Table 4.3 represent effects on OPRT activity. The purine nucleosides and the guanine nucleotides show relatively the highest inhibition.

4.4. Discussion

Our results indicate that the contributions of the pyrimidine de novo pathway and the salvage pathway differ markedly between the lymphocytes of the different species. In previous reports (370, chapter 2) we demonstrated that peripheral equine lymphocytes have very low OPRT and ODC activities and postulated that equine lymphocytes primarily depend on salvage pathways for pyrimidine nucleotide supply. Peripheral lymphocytes of pig seem to depend on the de novo synthesis for pyrimidine nucleotide supply, since with all pigs investigated (more than 25) high activities of OPRT and ODC were found (chapter 2 and Table 4.2 and 4.3). Furthermore uridine catabolism is more likely than phosphorylation in these lymphocytes since always a higher uridine phosphorylase activity was found. The variations of the activity of uridine kinase that were found in porcine lymphocytes suggest a heterogeneity for this enzyme in pigs.

At PHA-stimulation the salvage pathway seems to play a more important role for pyrimidine nucleotide synthesis than the de novo pathway. The activities of OPRT and ODC decrease with porcine lymphocytes, whereas Lucas (214) observed no change for human lymphocytes. Furthermore in human PHA-stimulated lymphocytes the increase in the activity of uridine kinase is higher than that of the first enzyme in pyrimidine de novo pathway, CPS and ATC (180, 192, 213, 214). In equine lymphocytes with their low OPRT and ODC activities, uridine kinase activity increased at PHA-stimulation, and in porcine lymphocytes the change in uridine kinase activity was inversely related to the activity at the start of the cultures.

The effect of PHA-stimulation on uridine phosphorylase activity was not previously reported. The higher increase and the higher absolute values of the uridine phosphorylase activity in porcine lymphocytes in comparison to in equine lymphocytes are compatible with the larger capacity of their pyrimidine nucleotide de novo pathway. In equine lymphocytes uridine phosphorylation appears to be essential for RNA synthesis. With intact peripheral equine lympho-

cytes no catabolism of uridine could be demonstrated (370).

Inhibition by CMP and UMP was found for ODC from various sources (41, 75, 116, 369, 376). The inhibition of OPRT by these nucleotides is mainly due to inhibition of ODC, that results in accumulation of OMP (364). The mechanism by which various nucleosides may inhibit the OPRT reaction in lysates of Ehrlich ascites cells (376) and lymphocytes is still not clear. PRPP depletion may be a cause, especially when it is present at a low concentration (278, chapter 7). A distinct effect of nucleosides on the orotic acid binding site of the enzyme may also be involved. The inhibition of OPRT by AMP, ADP, GMP, TTP, UTP was also reported for Ehrlich ascites cells (376) and/or human hemolysate (364, 369) but the inhibition by guanine nucleosides and nucleotides, except GMP, and SAH and SAM was not previously reported. Several of the tested compounds can accumulate in ADA or PNP deficiency (281). Therefore inhibition of pyrimidine nucleotide synthesis de novo by these compounds by their effects on OPRT and ODC activity or on PRPP synthesis and utilization (chapter 3) can not be excluded in the lymphocytes of these patients.

4.5. Summary

1. Activity of uridine kinase was very low in ovine lymphocytes and in those of some pigs. Lymphocytes of other pigs showed a significantly higher activity of this enzyme. Activity of uridine kinase in lymphocytes of man, horse and cattle was intermediate. 2. Activity of uridine phosphorylase was higher than that of uridine kinase with lymphocytes of all species. 3. Activity of uridine kinase in equine lymphocytes increases at PHA-stimulation and also in porcine lymphocytes with a low activity at the start of the culture. Activity of uridine kinase decreased in porcine lymphocytes with a high activity at the start of the culture. 4. Activity of uridine phosphorylase increases at PHA-stimulation with equine and porcine lymphocytes and during culturing of non-stimulated porcine lymphocytes. 5. Activities of OPRT and ODC decrease in cultures of porcine lymphocytes with and without PHA. 6. Activity of OPRT in lysates of porcine lymphocytes is inhibited by purine nucleosides and by guanine and pyrimidine nucleotides.

Chapter 5

ADENOSINE AND DEOXYADENOSINE METABOLISM IN MAMMALIAN LYMPHOCYTES*

5.1. Introduction

Three inherited enzyme deficiencies in purine catabolism in human lymphocytes are associated with severe dysfunction of the immune system. Agammaglobulinemia is associated with an ecto-5'-nucleotidase deficiency (93, 373), SCID with ADA deficiency (122) and a loss of T-cell function with PNP deficiency (123). The products and substrates of these enzymes, the purine nucleosides adenosine, guanosine, their nucleotides and deoxycompounds seem to play a key role in these deficiencies (281), but have also many other biological and biochemical actions (9, 109).

The metabolism of adenosine and deoxyadenosine is shown in Fig. 5.1. They can be formed by dephosphorylation of AMP and dAMP, a step catalyzed by phosphatases and nucleotidases, which are either cytosolic or bound to the outside of the plasma membrane (115, 224). Adenosine is also formed by hydrolysis of SAH which is a product of transmethylation reactions involving SAM. The backward reaction (formation SAH) is possible but will not be important under normal physiological conditions (162). Nucleoside kinases catalyze the phosphorylation of adenosine and deoxyadenosine (205, 233), ADA the deamination of adenosine and deoxyadenosine and PNP the phosphorylysis of inosine, guanosine and their deoxy-derivatives. Since adenosine and deoxyadenosine are rapidly transported across the cell membrane, their intracellular concentration will be regulated by adenosine metabolizing enzymes (218).

The precise mechanism by which ADA and PNP deficiencies are related to immune dysfunction is not known (281). A high extracellular concentration of adenosine, guanosine and their deoxy-compounds can influence the uptake of other nucleosides, who share the same transport system (218). Diminished concentrations of the products of ADA and PNP reactions and elevated concentrations of (d)nucleotides can lead to pyrimidine starvation (125), inhibition of PRPP synthesis (118) and disturbance in energy ratio (318). The high concentration

*adapted from Peters et al. (269)

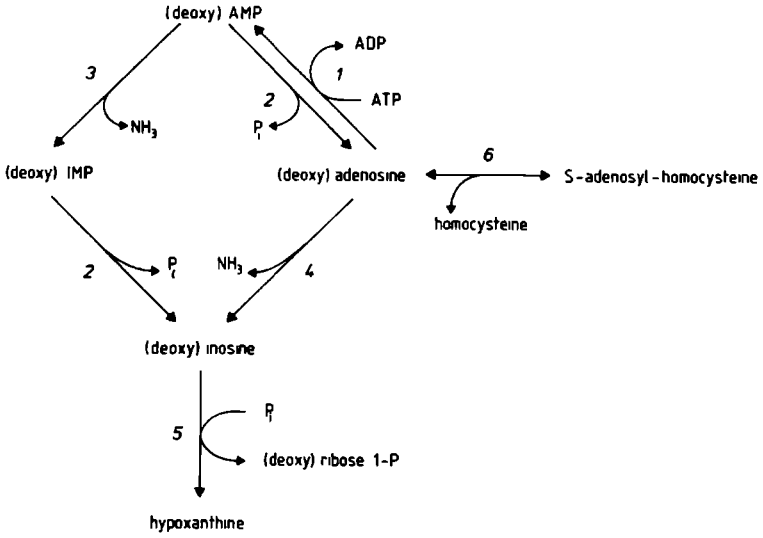


Fig. 5.1. Metabolism of adenosine and deoxyadenosine. The enzymes catalyzing the interconversions are: 1, nucleoside kinase; 2, 5'-nucleotidase; 3, AMP deaminase; 5, ADA; PNP and and 6, SAH hydrolase.

of dATP present in lymphocytes of patients with ADA deficiency (85) can inhibit ribonucleotide reductase activity resulting in an impairment of DNA synthesis. Accumulation of adenosine and deoxyadenosine can also elevate the concentration of SAH, a potent inhibitor of methylation reactions (144, 202).

Because patients with ADA deficiency and SCID suffer from a severe lymphopenia, most of the findings are based on studies of their erythrocytes, urine and plasma, and on studies at several model systems, including lymphoma cells and fibroblasts, transformed lymphoblasts and human PBL, either with or without inhibitors of ADA.

Earlier (368) we found that equine lymphocytes contain a low amount of ADA activity in the same order of magnitude as in lymphocytes of patients with ADA deficiency (261). Now we report large differences in ADA and PNP activities of lymphocytes and erythrocytes of 10 mammalian species. ADA activity was similarly low in equine and porcine lymphocytes and PNP activity in ovine and caprine lymphocytes was in the same range as in those of patients with PNP deficiency.

Knowledge of the adenosine and deoxyadenosine metabolism in lymphocytes of man and these mammals may give insight in the indispensable role of ADA and PNP in the immune function of human lymphocytes. A comparative study of maximum activities and kinetic properties of enzymes involved in adenosine metabolism will provide, as stated by Arch & Newsholme (8), useful information about the enzymatic basis for control of the changes in the concentrations of adenosine and deoxyadenosine and their 5'-monophosphate derivatives. Therefore we investigated in lymphocytes of man, horse, pig and sheep the activity of the adenosine and inosine producing enzymes 5'-nucleotidase, AMP deaminase and SAH hydrolase and the kinetic properties of the deaminase and kinase enzymes with adenosine and deoxyadenosine as substrates.

5.2. Materials and methods

5.2.1. Materials

Ficoll (MW 400 kD) was obtained from Pharmacia and Isopaque from Nyegaard & Co., Oslo, Norway. A Ficoll-Isopaque solution was prepared as described previously (370). {8-^{14}C}Adenosine, {U-^{14}C}-adenosine, {8-^{14}C}inosine, {8-^{14}C}adenosine monophosphate, {8-^{14}C}-inosine monophosphate and {U-^{14}C}uridine monophosphate were obtained from the Radiochemical Centre, Amersham. Aquasol and {8-^{14}C}deoxyadenosine were from the New England Nuclear Corporation, Boston, USA and plastic sheets coated with 0.1 mm PEI-cellulose from Merck. EHNA was a gift from Burroughs Wellcome Co., Research Triangle Park, NC, USA.

5.2.2. Animals

Blood samples were taken in heparinized bottles from healthy human volunteers and adult animals. The following species were used: man (*Homo sapiens*), horse (*Equus caballus*), pig (*Sus scrofa*), Texel sheep (*Ovis aries*), goat (*Capra hircus*), cattle (*Bos taurus*), Rhesus monkey (*Macaca familiaris*), white New Zealand rabbit (*Oryctolagus cuniculus*), Wistar rat (*Rattus norvegicus*) and Beagle dog (*Canis familiaris*). Blood samples from pig and cattle were obtained from the local slaughterhouse, equine, ovine and caprine blood from some local farmers.

5.2.3. Preparation of cell suspensions, lysates and extracts

Hemolysates were prepared from erythrocytes washed twice with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl (isotonic Tris-buffer). Cells were lysed by addition of a 3-fold volume of 10 mM Tris-HCl buffer (pH 7.4). For enzyme assays the hemolysates were diluted with 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA.

Lymphocytes were isolated by Ficoll-Isopaque centrifugation (38). Human, equine and monkey blood were centrifuged at 360 g; rat, rabbit and canine blood at 450 g and porcine, bovine, ovine and caprine blood at 700 g at 18°C for 30 min. The cells were washed twice with isotonic Tris buffer and counted in a hemocytometer. For enzyme assays on intact cells a suspension in isotonic Tris buffer was used after adjustment to the appropriate cell concentration. For cell extracts the cells were centrifuged, suspended in 50 mM Tris-HCl, containing 1 mM EDTA (pH 7.4) and lysed by sonication (Branson sonifier B12, 8 bursts of 5 sec at maximal output). For nucleoside kinase assays the lysate was centrifuged at 7000 g for 15 min at 4°C.

5.2.4. Enzyme assays

All enzyme activities were measured by radiochemical methods at 37°C in a shaking water-bath. Enzyme activities are expressed as nmols of product formed per hr per 10^6 cells and for erythrocytes as nmols of product formed per hr per mg protein. Linearity of the reactions with respect to time and amount of protein was ascertained. All reactions were terminated by heating for 2-5 min at 95°C in an Eppendorf heater. Excess carrier was added and, unless otherwise indicated, products and substrate were separated on PEI-cellulose thin-layers with distilled water. All nucleotides remained on the start position; nucleosides and bases were localized under u.v. The spots were cut out and eluted with 1.0 ml 0.1 M HCl/0.2 M KCl for 45 min. Radioactivity in eluates was always determined after addition of 10 ml Aquasol.

Protein content was determined according to Lowry et al. (212) with bovine serum albumin as standard.

5.2.5. ADA assay

The usual reaction mixture (60 µl) contained 50 mM Tris-HCl

buffer (pH 7.4), 1 mM EDTA, and hemolysate protein (60-3000 µg) or an amount of lymphocyte lysate equivalent to $2 - 30 \times 10^4$ cells. Reaction was started by addition of {8-^{14}C}adenosine (58 mCi/mmol, 0.13 mM final concentration, except otherwise given). Reaction was terminated after 10-30 min and as carriers adenosine, inosine and hypoxanthine were added. Separation was achieved by high-voltage paper-electrophoresis in acetic acid/formic acid/water (17/17/1000, by vol, pH 2.1) for 30 min at 40 V/cm. Spots were eluted with 5 ml of 0.1 M NaOH for 45 min.

5.2.6. PNP assay

The reaction mixture (50 µl) contained 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 80 mM phosphate (pH 7.4), 0.34 mM {8-^{14}C}inosine (7.3 mCi/mmol) and 10-2500 µg hemolysate protein or an amount of lymphocyte lysate equivalent to $3 - 25 \times 10^4$ cells. After 10 min the reaction was terminated and inosine and hypoxanthine were added as carriers. Quantitation of substrate and product was achieved as described under Enzyme assays.

5.2.7. 5'-nucleotidase and AMP deaminase assays

With UMP as a substrate the reaction mixture (60 µl) contained 33 mM Tris-HCl (pH 7.4), 0.7 mM EDTA, lymphocyte lysate (equivalent to $16 - 25 \times 10^5$ cells) and 2 mM {U-^{14}C}UMP (0.225 mCi/mmol). With {8-^{14}C}IMP (2.0 mM, 1.5 mCi/mmol) as a substrate 8.3 mM $MgCl_2$ was present. The activity with AMP as a substrate was measured at 0.12 mM {8-^{14}C}AMP (61 mCi/mmol) or at 2.0 mM {8-^{14}C}AMP (1.5 mCi/mmol), in the presence of 4.2 mM $MgCl_2$. Dephosphorylation of nucleotides by aspecific phosphatases was inhibited by 15 mM glycerol 2-P, when indicated. After 30-90 min the reaction was terminated and carriers were added (AMP, IMP, ADP, ATP, adenosine, inosine and hypoxanthine for purine 5'-nucleotidase and UMP, uridine and uracil for pyrimidine 5'-nucleotidase assay). For the 5'-nucleotidase assays with UMP, IMP and 0.12 mM AMP as substrates quantitation of substrates and products was achieved as described above.

Since in the incubation mixture with 2.0 mM AMP the activity of AMP deaminase was also assayed IMP had to be separated from AMP. The method described by Reibel & Rovetto (300) was modified. First the PEI-cellulose thin-layer was developed with a mixture of butanol/methanol/water (1/1/8, by vol.) and dried. The adenosine spots were

localized under u.v. The sheet was developed thereafter with 1.4 M LiCl until the adenosine spot. The nucleotides and nucleosides were quantitated as described above.

The activty of ecto-5'-nucleotidase was determined with 0.12 mM {8-^{14}C}AMP (61 mCi/mmol) or 0.12 mM {8-^{14}C}IMP (61 mCi/mmol) as substrates. Further the mixture contained 33 mM Tris-HCl (pH 7.4), 108 mM NaCl, 4.2 mM $MgCl_2$ and 4 - 20 x 10^5 cells. After 30-60 min incubation in a vigorously shaking water-bath the reaction was terminated by heating for 5 min at 95°C. Quantitation of substrate and products was achieved as described above. In parallel incubations the reaction was terminated by spinning down the cells at 14 000 g for 1 min. The cells were washed with 40 µl isotonic Tris solution containing excess of carriers. The pellet was suspended in 70 µl of a solution containing 1.1 M formic acid and carriers. Substrate and products were quantitated as described under Enzyme assays.

5.2.8. Nucleoside kinase assays

The standard incubation mixture (80 µl) contained 30 mM Tris-HCl buffer (pH 7.4), 0.625 mM EDTA, 2.5 mM ATP, 1.1 mM $MgCl_2$, 62.5 µM EHNA, a volume of 7000 g supernatant equivalent to 20 - 35 x 10^5 cells and 50 µM {8-^{14}C}adenosine (58 mCi/mmol). To increase sensitivity and to avoid excessive consumption of the substrate we used an amount of supernatant equivalent to 10 - 25 x 10^3 cells and varying concentrations of {U-^{14}C}adenosine (559 mCi/mmol)for the determination of the Km value for adenosine. For assay of the Km for deoxyadenosine a supernatant volume equivalent to 20 - 35 x 10^5 cells and {8-^{14}C}deoxyadenosine at varying specific activities were used. After incubation for 10-30 min (with adenosine) or 60-90 min (with deoxyadenosine) the reaction was terminated by heating and addition of excess AMP, adenosine and inosine or their deoxy-compounds. Quantitation of substrates and products was achieved as described under Enzyme assays.

5.2.9. SAH hydrolase assay

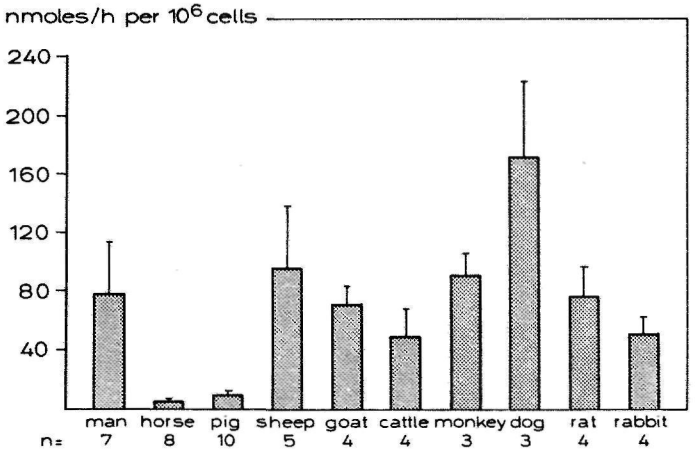
The reaction mixture (55 µl) contained 25 mM Tris-HCl (pH 7.4), 0.45 mM EDTA, 91 µM EHNA, 8.2 mM DL-homocysteine, and hemolysate protein (800-1000 µg) or an amount of lymphocyte lysate equivalent to 10 - 18 x 10^5 cells. Reaction was started by addition of {8-^{14}C}-adenosine (58 mCi/mmol, 0.15 mM final concentration) or {8-^{14}C}-

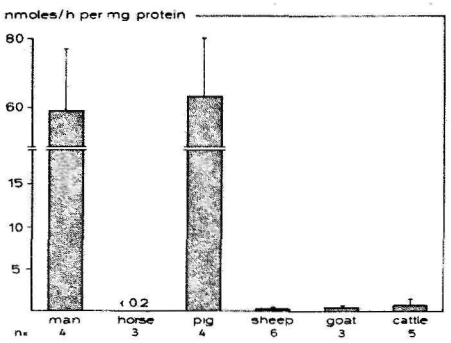
deoxyadenosine (8.2 mCi/mmol, 0.23 mM final concentration). After 10 min the reaction was terminated by heating and addition of carrier adenosine, inosine, SAH, SAM and AMP. Quantitation of substrate and products was achieved as described under Enzyme assays.

5.3. Results

5.3.1. ADA and PNP

In ADA assays the main products were inosine and deoxyinosine. When a considerable PNP activity was present hypoxanthine was also formed. Addition of EDTA and the absence of ATP prevented formation





↑ Fig. 5.2. Activity of ADA in lysates of mammalian lymphocytes. Enzyme activities (in nmol/hr per 10^6 cells) are means ± SD for the number of indiduals indicated by n.

← Fig. 5.3. Activity of ADA in mammalian hemolysates. Enzyme activities (in nmol/hr per mg protein) are means ± SD for the number of individuals indicated by n.

of AMP. Data for ADA and PNP in man, horse and cattle were previously reported (368), but are included for comparison with those of other species and because more values were obtained. ADA activities in lymphocytes and erythrocytes are shown in Figs. 5.2 and 5.3, respectively, and PNP activities in Figs. 5.4 and 5.5, respectively. ADA activities in the lymphocytes of seven species are comparable to that of human lymphocytes, but the activities in lymphocytes of horse and pig are very low. PNP activity is relatively low in lymphocytes of sheep and goat, high in human lymphocytes and intermediate in those of seven other mammalian species.

The activities of ADA and PNP in hemolysates of the various

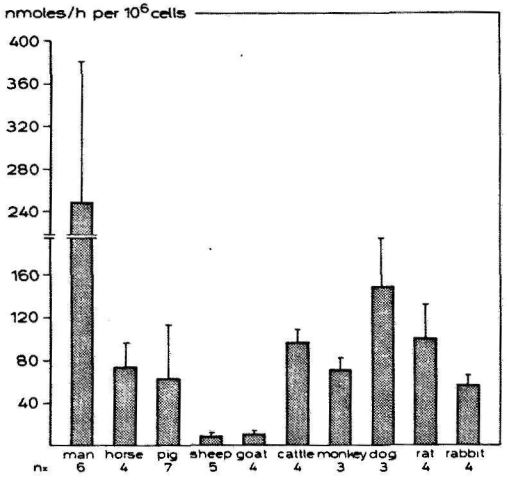


Fig. 5.4. Activity of PNP in lysates of mammalian lymphocytes. Enzyme activities (in nmol/hr per 10^6 cells) are means ± SD for the number on individuals indicated by n.

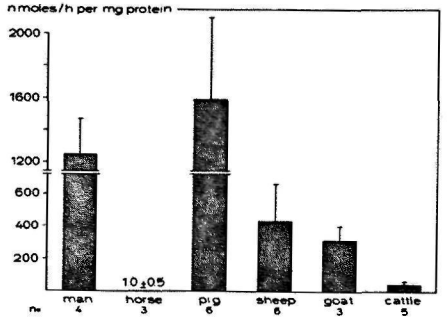


Fig. 5.5. Activity of PNP mammalian hemolysates. Enzyme activities (in nmol/hr per mg protein) are means ± SD for the number of individuals indicated by n.

mammals show large variations and do not always correlate with those in lymphocytes of the same species. In horse both in erythrocytes and lymphocytes activity of ADA is low. Activities of ADA are relatively low in hemolysates of sheep, goat and cattle, in contrast to in their lymphocytes. In hemolysates of man and pig the activities of ADA are comparable, in contrast to the activities in their lymphocytes. Activities of PNP are low in erythrocytes of cattle and horse, in contrast to in their lymphocytes. On the other hand, PNP activity is considerable in ovine and caprine hemolysates, in contrast to in their lymphocytes.

We also measured ADA activity with deoxyadenosine as a substrate and determined the Km values with a Lineweaver-Burke plot for adenosine and deoxyadenosine in the lymphocytes of man, horse and pig (Table 5.1). In the same species ADA activity is similar with adenosine and deoxyadenosine. Km values for adenosine and deoxyadenosine are equal in lymphocytes of the same species. In equine lymphocytes, however, the affinity of adenosine and deoxyadenosine is higher than in those of man and pig.

Table 5.1. Km values and activities of ADA with adenosine and deoxyadenosine as substrates in lysates of mammalian lymphocytes

Species	Activity		Km	
	Adenosine	Deoxyadenosine	Adenosine	Deoxyadenosine
Man	79 ± 36 (7)	82 ± 29 (4)	32.9 ± 8.8 (4)	25.1 ± 4.2 (4)
Horse	5.1 ± 2.0 (8)	6.8 ± 2.3 (6)	9.7 ± 3.7 (4)	10.4 ± 4.3 (4)
Pig	9.9 ± 3.4 (10)	14.1 ± 3.6 (4)	37.0 ± 6.2 (3)	28.7 ± 4.4 (4)

Activities (in nmol/hr per 10^6 cells) and Km values (in μM) are means ± SD for the number of individuals given in parentheses.

5.3.2. 5'-nucleotidases

Adenosine formation from AMP can be catalyzed by 5'-nucleotidases and aspecific phosphatases. To determine the 5'-nucleotidase activity we added glycerol 2-P to inhibit aspecific dephosphorylation (200). Dephosphorylation was studied in whole lysates of lymphocytes at 2 mM AMP, IMP and UMP to determine substrate specificity. AMP was also used at 0.12 mM to compare the total cellular acti-

Table 5.2. Activity of 5'-nucleotidase with different substrates in the presence and absence of glycerol 2-P (G 2-P) in lysates of mammalian lymphocytes

Species	Addition of G 2-P	AMP		IMP	UMP
		0.12 mM	2 mM	2 mM	2 mM
Man	-	5.2 ± 2.9 (9)	18.2 ± 5.7 (6)	18.9 ± 4.4 (6)	5.0 ± 1.8 (7)
	+	4.7 ± 2.5 (8)	15.5 ± 4.4 (6)	17.5 ± 3.1 (5)	3.8 ± 0.9 (4)
		94 ± 8 (8)	87 ± 16 (6)	92 ± 11 (5)	70 ± 17 (4)
Horse	-	1.2 ± 0.7 (11)	4.0 ± 2.2 (6)	4.8 ± 2.4 (6)	3.9 ± 1.2 (5)
	+	0.2 ± 0.1 (11)	2.0 ± 0.9 (6)	1.8 ± 0.5 (4)	1.6 ± 0.7 (4)
		18 ± 9 (10)	43 ± 15 (6)	56 ± 22 (4)	42 ± 16 (4)
Pig	-	0.5 ± 0.2 (9)	2.4 ± 1.7 (6)	5.6 ± 3.8 (11)	2.6 ± 1.3 (13)
	+	0.2 ± 0.1 (7)	1.4 ± 0.7 (6)	3.0 ± 1.0 (7)	1.4 ± 1.1 (6)
		63 ± 17 (7)	60 ± 11 (6)	92 ± 33 (7)	50 ± 18 (6)
Sheep	-	3.0 ± 1.7 (6)	8.3 ± 1.7 (7)	8.8 ± 2.5 (6)	3.2 ± 0.9 (5)
	+	1.8 ± 0.8 (5)	6.7 ± 1.3 (7)	7.3 ± 1.5 (4)	1.5 ± 0.5 (4)
		73 ± 17 (5)	82 ± 11 (7)	89 ± 21 (4)	45 ± 12 (4)

Activities (in nmol/hr per 10^6 cells) are means ± SD for the number of individuals indicated in parentheses. The effect of glycerol 2-P (15 mM) is given in % as the mean ± SD of relative activity in the absence of glycerol 2-P.

vity at this concentration with that of membrane-associated 5'-nucleotidase, which was measured on intact lymphocytes. This low concentration was used because of the high affinity of the ecto-enzyme for AMP (115).

Dephosphorylation of IMP and AMP needed the presence of $MgCl_2$ (data not shown). 5'-Nucleotidase activity was always higher at 2 mM than at 0.12 mM AMP and in the same order of magnitude for IMP (Table 5.2). UMP was a poorer substrate in lysates of all species except horse. Activity of purine 5'-nucleotidase was highest in human lymphocytes and markedly lower in equine and porcine lymphocytes. Omission of glycerol 2-P did not markedly change this pattern. In man omission of glycerol 2-P did not influence dephosphorylation of both purine nucleotides. Dephosphorylation rates of all purine nucleotides in equine lymphocytes and that of AMP in porcine lymphocytes were markedly higher in the absence of glycerol 2-P. In the animal species the contribution of aspecific phosphatases in dephos-

phorylation of UMP was relatively high.

No difference was found in the dephosphorylation rates by intact lymphocytes after termination of the reaction by heating or spinning down the cells. When we had confirmed this for each species the heating procedure was used in all further assays. The method with centrifugation did not give information on a possible transport function of an ecto-5'-nucleotidase, because the cells were thoroughly washed and a low specific activity was used. Cell viability tested at the end of incubation by trypan blue exclusion was more than 95% in all species. Dephosphorylation rate of AMP was higher with intact human lymphocytes than with equine and porcine lymphocytes (Table 5.3), as was also found with lysates (Table 5.2). Moreover the dephosphorylation rate with intact lymphocytes of horse and pig was (for 71 and 49%, respectively, means of 4 experiments) due to an aspecific ecto-phosphatase activity, as was found by addition of 15 mM glycerol 2-P to the incubation mixture. With intact human lymphocytes glycerol 2-P had no effect. With intact human lymphocytes of the same individual AMP was a better substrate than IMP. The ratio of the activities was 1.56 ± 0.11 (mean ± SD of 3 determinations). At equimolar concentrations (0.12 mM) AMP dephosphorylation was inhibited by IMP with 29 ± 15% (mean ± SD of 3 experiments).

Table 5.3. Dephosphorylation rates of AMP and IMP with intact mammalian lymphocytes

Species	AMP	IMP
Man	6.04 ± 2.73 (7)	5.49 ± 1.91 (3)
Horse	0.44 ± 0.22 (5)	N.D.
Pig	0.19 ± 0.15 (8)	N.D.

Acitvities (in nmol/hr per 10^6 cells) are means ± SD for the number of individuals indicated in parentheses. Concentration of nucleotides was 0.12 mM. N.D., not determined.

5.3.3. AMP deaminase

AMP can also be degraded by deamination. We measured the activity of AMP deaminase simultaneously with dephosphorylation of AMP at a concentration of 2 mM, although this concentration is suboptimal for deamination. Total substrate consumption for the two reactions did not exceed 10% of the initial concentration. Addition of

glycerol 2-P did not influence deamination. To ascertain that the product inosine was only formed via dephosphorylation and subsequently deamination of adenosine in some assays 80 µM EHNA, an inhibitor of ADA was added to the incubation mixture. An amount of radioactivity equal to that at the inosine spot in the incubation mixture without EHNA, was now found additionally at the adenosine spot. The amount of radioactivity found at the IMP spot was sometimes lower in the presence of EHNA, which may be caused by inhibition of AMP deaminase (136). Therefore EHNA was omitted from the standard assay. Since inosine was only formed by dephosphorylation followed by deamination of adenosine, the activity of AMP deaminase could be calculated from the amount of IMP formed. The activity of AMP deaminase was considerable in human and porcine lymphocytes, but low in equine and ovine lymphocytes (Table 5.4).

Table 5.4. Activity of AMP deaminase in lysates of mammalian lymphocytes

Species	Activity
Man	11.5 ± 6.5
Horse	0.9 ± 0.7
Pig	11.8 ± 3.4
Sheep	1.1 ± 0.9

Activities (in nmol/hr per 10^6 cells) are means ± SD for 4-5 individuals. Concentration of AMP was 2 mM.

5.3.4. Nucleoside kinase(s)

Adenosine and deoxyadenosine formed from AMP and dAMP, respectively can either be deaminated by ADA or phosphorylated by a kinase. The optimal concentrations of ATP and $MgCl_2$ for the latter reaction are disputed (7, 56, 233). Therefore we first determined these concentrations for our assay. A combination of 2.5 mM ATP and 0.5 mM $MgCl_2$ gave the highest activity. $MgCl_2$ concentration is corrected for the presence of EDTA. EHNA was added to prevent deamination of adenosine and deoxyadenosine. To ascertain that EHNA had no effect on kinase activity it was omitted in some assays with equine lymphocytes. No difference was found. Kinase activities with adenosine as a substrate (Table 5.5) represent values determined at a concentration of 50 µM adenosine with a high protein concentration and Vmax values obtained in kinetic studies with low protein concentrations and calculated from Lineweaver-Burke plots. The activities of adenosine kinase were comparable in extracts from lymphocytes of man, pig,

Table 5.5. Activities and Km values of nucleoside kinase(s) for adenosine and deoxyadenosine in extracts of mammalian lymphocytes

Species	Activity		Km	
	Adenosine	Deoxyadenosine	Adenosine	Deoxyadenosine
Man	1.50 ± 0.99 (8)	0.26 ± 0.15 (3)	3.44 ± 0.85 (4)	76 ± 22 (5)
Horse	1.80 ± 0.22 (8)	0.54 ± 0.19 (4)	0.94 ± 0.30 (3)	490 ± 105 (4)
Pig	0.72 ± 0.30 (6)	0.20 ± 0.14 (8)	0.80 ± 0.12 (3)	504 ± 156 (8)
Sheep	1.17 ± 0.47 (5)	N.D.	N.D.	N.D.

Activities (in nmol/hr per 10^6 cells) and Km values (in µM) are means ± SD for the number of individuals given in parentheses.

horse and sheep. Activity with deoxyadenosine (all calculated as Vmax values) was lower in the same species. With small amounts of lymphocyte extracts (equivalent to 10 - 25 x 10^3 cells per assay) substrate inhibition was observed above 5 µM adenosine. With large amounts of lymphocyte extracts (equivalent to 20 - 35 x 10^5 cells per assay) this substrate inhibition was not observed until 100 µM. Deoxyadenosine did not show substrate inhibition. Km values for adenosine were markedly lower than values for deoxyadenosine. In human lymphocytes the Km value for adenosine was higher than in equine and porcine lymphocytes, while the Km for deoxyadenosine was lower.

Adenosine is generally assumed to be phosphorylated by adenosine kinase (109). Phosphorylation of deoxyadenosine may be catalyzed by more enzymes including adenosine kinase (7, 49, 234). There-

Table 5.6. Influence of adenosine and deoxycytidine on deoxyadenosine phosphorylating activity in mammalian lymphocytes.

Species	Relative activity in the presence of	
	Adenosine	Deoxycytidine
Man	97 ± 12	31 ± 9
Horse	55 ± 6	87 ± 3
Pig	79 ± 6	61 ± 8

Compounds were added at equimolar concentrations (67 µM) of deoxyadenosine. Values (in % of the activity without addition) are means ± SD of 3 experiments.

fore we determined the infuence of adenosine and deoxycytidine on the phosphorylation of deoxyadenosine (Table 5.6). In extracts of human lymphocytes adenosine had no influence while deoxycytidine considerably inhibited the phosphorylation of deoxyadenosine. With equine and porcine lymphocytes deoxycytidine had a smaller effect but adenosine inhibited markedly.

5.3.5. SAH hydrolase

Another enzyme that can metabolize adenosine is SAH hydrolase. Optimal substrate concentrations were determined for adenosine and homocysteine. Activity in human hemolysate was 3.2 ± 0.5 nmol/hr per mg protein (mean ± SD of 3 experiments). With adenosine as a substrate activity in lysates of human lymphocytes was lower than in those of horse and pig (Table 5.7). With deoxyadenosine no activity was detectable in lymphocyte lysate. Deoxyadenosine inhibited, however, hydrolase activity if it was added to the incubation mixture 15 min before addition of adenosine (Table 5.8). When deoxyadenosine and adenosine were added simultaneously no effect of deoxyadenosine was observed.

Table 5.7. Activity of SAH hydrolase in lysates of mammalian lymphocytes

Species	Activity
Man	1.6 ± 1.2 (8)
Horse	2.4 ± 1.3 (9)
Pig	2.4 ± 1.1 (8)

Activities (in nmol/hr per 10^6 cells) are means ± SD for the number of expe- experiments given within parentheses.

5.4. Discussion

Activities of ADA and PNP in human lymphocytes and erythrocytes are comparable to values reported by other authors (155, 261, 400). With a histochemical assay Borgers & Thone (34) found high PNP activities in lymphocytes of man, rabbit and dog, moderate activity in those of rat and a low activity in those of pig. ADA activities, measured by McGuire et al. (229) in lymphocytes of normal horses and of foals with SCID, did not differ and were similar to our value. No more comparative data on ADA and PNP activities in mammalian lymphocytes were found.

No significant ADA activity was observed in equine hemolysate

Table 5.8. Effect of deoxyadenosine on the activity of SAH hydrolase in lysates of lymphocytes of man, horse and pig

Species	Relative activity in the presence of deoxyadenosine	
	during incubation	during preincubation and incubation
Man	94 ± 4	57 ± 8
Horse	95 ± 7	16 ± 2
Pig	103 ± 1	51 ± 6

Concentration of deoxyadenosine was 167 µM. After 15 min preincubation, incubation was started by addition of 150 µM adenosine. Values (in % of the activity without deoxyadenosine) are means ± SD of 3 experiments.

by McGuire et al. (229) in contrast to Castles et al. (50), who also reported a higher PNP activity. The high adenosine disappearance rate for erythrocytes of man and pig and the low for those of cow, sheep and goat reported by Van Belle (396) correlate with the large variation in ADA activity. Duhm (90) observed a similar order for the PNP activity in hemolysates of man>pig>sheep>beef, but did not report values for goat and horse. The high ADA and PNP activities of porcine red blood cells may relate to their use of inosine as energy source (407).

ADA activities in equine and porcine lymphocytes are comparable to the activity found in those of patients with SCID associated with ADA deficiency (155, 261). Furthermore PNP activities in ovine and caprine lymphocytes approximate the values in patients with PNP deficiency (315, 332). Therefore equine and porcine lymphocytes and ovine and caprine lymphocytes could provide useful model systems to elucidate the indispensable role of ADA and PNP, respectively, in human lymphocytes. Our simultaneous measurements of ADA and PNP activities in lymphocytes and erythrocytes of the same species show that the activities of ADA and PNP in hemolysates do not correlate in most mammals with the activity in lymphocytes. In man there is a correlation in ADA and PNP activities between lymphocytes and erythrocytes, but still an ADA deficiency in erythrocytes is not always coupled with absence of lymphocyte ADA activity and SCID (155).

Km values of adenosine for ADA in the lymphocytes of man, horse

and pig are in the range reported for erythrocytes (2, 233), rat liver (182), lymphoid cell lines (155) and various tissues of several species (8). Deoxyadenosine degradation was as rapid as that of adenosine as was earlier found in lymphoid cells (155) and for various tissues of six mammals (39). Equine lymphocytes appear to be able to degrade more efficiently adenosine and deoxyadenosine than those of pig, because of the relatively low Km values for ADA.

Activities of 5'-nucleotidase assayed in lysates of human lymphocytes at 0.2 mM AMP (92, 93) were similar to our values. Also the ecto-5'-nucleotidase activities measured with either AMP or IMP (66, 93, 95, 373, 411) are comparable with our results. No data are available on 5'-nucleotidase activities in the peripheral lymphocytes of horse, pig and sheep. A glycerol 2-P inhibitable activity of a-specific ecto-phosphatase is present on equine and porcine lymphocytes, as was earlier found for rat lymphocytes (247). By the low activity of 5'-nucleotidase in and on equine and porcine lymphocytes little adenosine and deoxyadenosine will be formed, which may prevent accumulation of these nucleosides.

The other breakdown route of AMP via IMP and inosine is also not important in lymphocytes of horse, pig and sheep, because in their lymphocytes one of the enzymes necessary for this pathway has a very low activity. This route will also not be important in human lymphocytes, since the Km value of AMP is much lower for 5'-nucleotidase (93) than for AMP deaminase (397).

Activity of AK in human lymphocytes is lower than the value reported by Snyder et al. (343) and can not be compared with the value of Carson et al. (46) who measured activity in undefined extracts. The latter authors found a comparable ratio of the phosphorylation rates af adenosine and deoxyadenosine. No data are available on the activities of kinases in lymphocytes of the other species. The Km values of adenosine for AK in the mammalian lymphocytes are well comparable to the values reported for rat liver (182), rabbit liver (234), human hemolysate (233), L1210 cells (56) and for several tissues of various species (8). Apparent Km values of deoxyadenosine for phosphorylation by extracts from different sources (109, 205) are similar to those observed by us in extracts of equine and porcine lymphocytes, but higher than that in human lymphocytes. We established the results of Carson et al. (49) that phosphorylation of deoxyadenosine in human lymphocyte extracts was inhibited by deoxycytidine and not by adenosine. AK will not play an important role in the phosphorylation of deoxyadenosine, but a deoxycytidine kinase

and possibly a deoxyadenosine kinase will do (49, 205). In contrast, in equine and porcine lymphocytes AK appears also to phosphorylate deoxyadenosine like in murine lymphocytes (49).

The activity of SAH hydrolase in human hemolysate was similar to the value reported by Hershfield et al. (144). No data are available on activities in lymphocytes of the three species. The inhibition of SAH hydrolase by deoxyadenosine was time-dependent and not reversible, as was previously found in human lymphoblasts and erythrocytes (144, 202). Because deoxyadenosine is not converted to deoxyadenosyl-homocysteine, as was also found by Zimmerman at al. (432) for murine lymphocytes, it will irreversibly be bound to the enzyme, resulting in an accumulation of SAH. In the presence of high concentrations of adenosine this accumulation will also occur. The accumulated SAH inhibits in both cases methylation reactions in which SAM is involved.

The observed differences in adenosine and deoxyadenosine metabolism between human blood cells on one hand and porcine and equine blood cells on the other hand may explain why lymphocytes of horse and pig show a normal immune function despite a low ADA activity. Low amounts of adenosine and deoxyadenosine may be formed in equine and porcine lymphocytes, because of the low 5'-nucleotidase activity. In equine lymphocytes the low ADA activity can be used efficiently because the enzyme has a rather high affinity for the substrate. Equine lymphocytes are still more sensitive than human lymphocytes for inhibition of mitogen-induced blastogenesis by adenosine (211, chapter 7). In the pig the red blood cells contain a rather high ADA activity and may take up nearly all adenosine to deaminate it. The same principle is used in therapy with irradiated erythrocytes (280).

Since deoxyadenosine shows a higher Km value for phosphorylation than for deamination and its phosphorylation rate is low, deoxyadenosine flux will be directed to deoxyinosine in all species. In human lymphocytes, however, deoxyadenosine shows a relatively lower Km value for phosphorylation. Therefore dAMP will be formed at lower deoxyadenosine concentrations than in equine and porcine lymphocytes. Furthermore in man adenosine does not inhibit deoxyadenosine phosphorylation in contrast to in equine and porcine lymphocytes. This phenomenon may contribute to the high deoxyadenosine-nucleotide concentrations in lymphocytes of patients with ADA deficiency and SCID (85) in spite of their high adenosine concentration (206).

From the observed variations in adenosine and deoxyadenosine metabolism between human and equine and porcine lymphocytes it is

still not clear by which mechanism ADA deficiency in man can lead to SCID. The high concentration of deoxyadenosine nucleotides in lymphocytes of patients with ADA deficiency and SCID (85) can inhibit the ribonucleotide reductase activity. Synthesis of PRPP which is enhanced in transforming cells can be inhibited by (d)nucleotides (118, chapter 3). Such an accumulation does not seem probably in lymphocytes of horse and pig despite a low ADA activity, because of the observed differences in adenosine and deoxyadenosine metabolism. Evidence is now given that in lymphocytes deoxyadenosine may cause inhibition of methylation reactions.

In spite of the observed differences equine and porcine lymphocytes may form good model systems for studying the effect of exposure of lymphocytes with low ADA activity to high concentrations of adenosine and deoxyadenosine. Investigation of the mitogen-induced blastogenesis under these conditions may give more insight in the complicated mechanism by which ADA deficiency is related to disturbances of immune function in man.

5.5. Summary

1. Large differences were found in activities and kinetics of adenosine and deoxyadenosine metabolizing enzymes in lymphocytes of man and various mammalian species. 2. ADA and PNP activities in erythrocytes do not always correlate with those in lymphocytes of the same species. 3. ADA activity was low in equine and porcine lymphocytes, PNP activity was low in ovine and caprine lymphocytes. Adenosine and deoxyadenosine showed comparable Km values for ADA in lymphocytes of man, horse and pig; in equine lymphocytes these values were lower. 4. Purine 5'-nucleotidase activity was low in lysates of lymphocytes of horse and pig, intermediate in those of sheep and high in those of man. The same pattern was found for ecto-5'-nucleotidase. 5. Adenosine was phosphorylated at a higher rate than deoxyadenosine in lymphocyte extracts of man, horse and pig. Activities are comparable in all species. The Km value of adenosine for AK is higher but the Km of deoxyadenosine for phosphorylation lower in human lymphocytes than in lymphocytes of horse and pig. Deoxycytidine markedly inhibited deoxyadenosine phosphorylation in human lymphocytes and to a lower extent in equine and porcine lymphocytes. Adenosine considerably inhibited deoxyadenosine phosphorylation in lymphocytes of horse and pig, while it han no effect in human lymphocytes. 6. Activity of SAH hydrolase, measured in the synthetic

direction, was lower in human lymphocytes than in those of pig and horse. Deoxyadenosine markedly inhibited enzyme activity only if it was preincubated with the enzyme without adenosine. 7. The observed differences in adenosine and deoxyadenosine metabolism between human lymphocytes and those of horse and pig may explain why equine and porcine lymphocytes show a normal immune fuction despite a low ADA activity.

Chapter 6

STIMULATION BY PHA OF PBL FROM HORSE, PIG, SHEEP AND MAN*

6.1. Introduction

PHA is a widely used mitogen that stimulates thymus-derived lymphocytes to divide (211). Several metabolic studies have been conducted on PHA-stimulated mammalian lymphocytes. However, comparison of their results is difficult since various incubation conditions (e.g. different media and sera) have been used. In our studies on the effects of purine nucleosides on lymphocyte function and metabolism, we suggested that lymphocytes of horse, pig and sheep coul be used as possible model systems for ADA and PNP deficiency (403, chapter 5). Therefore we had to develop comparable systems for stimulation of lymphocytes from these species and from man, since comparison of results could be troubled by using different incubation conditions. In this study we report optimal cultivation conditions for transformation of mammalian lymphocytes by PHA in MEMS medium supplemented with horse serum.

6.2. Materials and methods

6.2.1. Materials

Ficoll (MW 400 kD) was obtained from Pharmacia and Isopaque fro Nyegaard & Co., Oslo, Norway. A Ficoll-Isopaque solution was prepare as described previously (370). {6-^{3}H}Thymidine was obtained from the Radiochemical Centre, Amersham, UK and Phytohemagglutinin-P was from Difco Laboratories. MEMS medium, RPMI 1640 medium, horse serum (Mycoplasma screened), swine serum, lamb serum and human serum (pooled) were from Flow Laboratories, Irvine, UK. Soluene-100 and hydroxide of Hyamine 10-X were from Packard, Groningen, the Netherlands, and Protosol form New England Nuclear, Dreieichenhain, FRG.

6.2.2. Animals

Blood samples were taken in heparinized bottles from healthy

*adapted from Peters & Veerkamp (272)

human volunteers and adult horses (Equus caballus), pigs (Sus scrofa) and Texel sheep (Ovis aries).

6.2.3. Preparation and culture of lymphocytes

Lymphocytes were isolated with a Ficoll-Isopaque gradient centrifugation (38) modified as described in chapter 5. Cells were counted in a hemocytometer and suspended in the appropriate medium. The trypan blue test showed a viability of more than 95%. With all species a purity of more than 95% mononuclear cells was found as was determined with May-Grünwald-Giemsa staining.

All cultures were performed at least in quadruplicate in 0.2 ml U-shaped microtitration 96-well plates (Flow Laboratories, Irvine,UK) under an atmosphere of 95% air/5% CO_2 at 37°C and 90-95% humidity. Bicarbonate-buffered MEMS medium (21.3 mM $NaHCO_3$) was used, unless otherwise indicated, and all media were supplemented with 2 mM L-glutamine, 100 µg streptomycin and 100 Units penicillin per ml. At 45 or 69 hr 2.6 µM {6-^{3}H}thymidine (3.3 Ci/mmol) was added and after 6 hr the cells were isolated with a Titertek Cell Harvester (Flow Laboratories, Irvine, UK). DNA was precipitated with 5% TCA. The glass-fiber filters were dried (15 min at 40°C) and 0.2 ml of Soluene-100 and 5 ml of toluene containing 6 g PPO and 0.4 g dimethyl-POPOP per l were added. Radioactivity was measured in a Packard PRIAS Tri-Card Liquid Scintillation Counter.

6.3. Results

Concentration of thymidine was optimized with respect to the amount of radioactivity and of unlabelled thymidine in order to prevent exhaustion of the thymidine pool and to minimize internal radiation. Incorporation of thymidine was linear with time for at least 6 hr with lymphocytes of all species studied and maximal at 2.6 µM thymidine. Of several counting systems tested a toluene solution of PPO and POPOP gave the best reproducible results. Before counting the filters had to dried and eluted with 0.2 ml Soluene-100. Wet filters disturbed the liquid-scintillation counting. Other strong bases like Hyamine and Protosol gave coloured solutions that suppressed counting efficiency.

With lymphocytes of all species several PHA concentrations were tested at various cell concentrations and with different amounts and several kinds of sera. Fig. 6.1 shows dose-response curves for

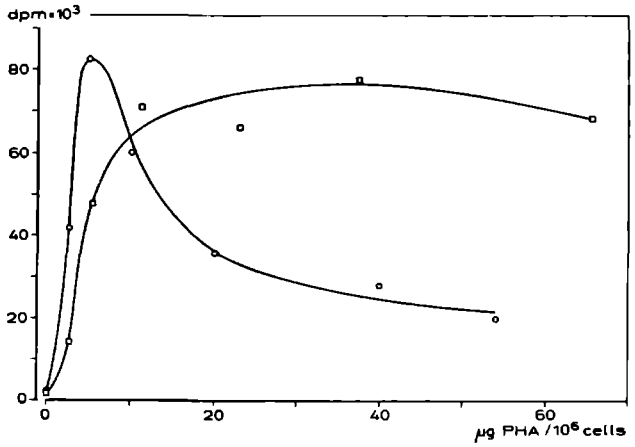


Fig. 6.1.
Dose-response curves to PHA ovine (□) and porcine (o) lymphocytes. The curves are representative out of 5 and 3 experiments, respectively.

PHA for ovine and porcine lymphocytes under optimal cultivation conditions. With human lymphocytes thymidine incorporation was linear at cell concentrations from 0.5 - 2 x 10^6 cells per ml and optimal at a concentration of 10% horse serum. With horse serum a higher thymidine incorporation was obtained than with human serum. With equine lymphocytes thymidine incorporation was linear at cell concentrations from 1 - 2 x 10^6 cells per ml; a higher thymidine incorporation was obtained with 20% horse serum than with 10%. Porcine lymphocytes showed a linear relationship between cell concentration and thymidine incorporation at 1 - 2 x 10^6 cells per ml. 10% Horse serum gave better results than 20% horse serum or several concentrations of swine serum.

Table 6.1. Response of ovine lymphocytes to PHA in various conditions

Serum	PHA	MEMS medium	RPMI medium
10% horse	-	3407	9010
	+	78980	77591
20% horse	-	5259	10255
	+	51399	89768
10% lamb	-	4301	22164
	+	16024	79061
20% lamb	-	5146	16784
	+	43237	50718

Values represent dpm of {6-^{3}H}thymidine incorporated into DNA at 51 hr for one representative experiment in quadruplicate. Comparable data were obtained in four separate experiments. Concentration of PHA was 10 µg/10^6 cells and that of the cells was 2 x 10^6 cells/ml.

Table 6.2. Thymidine incorporation at 51 hr of PHA-stimulated lymphocytes of man, horse, pig and sheep under optimal culture conditions

Species	PHA conc. ($\mu g/10^6$ cells)		Thymidine incorporation	
			Control	PHA
Man	20	Mean (9)	892 ± 416	69076 ± 48242
		Range	105 - 1489	29269 - 168103
Horse	1.25	Mean (14)	2119 ± 874	44057 ± 13153
		Range	487 - 3600	11971 - 65247
Pig	5	Mean (18)	4309 ± 2483	115629 ± 52123
		Range	584 - 7692	37524 - 245310
Sheep	25	Mean (7)	2768 ± 793	59936 ± 36994
		Range	1783 - 4161	23037 - 130780

Values represent dpm of {6-^{3}H}thymidine incorporated per 0.2 ml and are means ± SD for the number of experiments indicated within parentheses. The range is also given. Cell concentrations are 0.5×10^6/ml for human and 2×10^6/ml for animal lymphocytes.

For ovine lymphocytes we used several concentrations of medium, serum and percentages of serum in the experiments. In Table 6.1 thymidine incorporation is shown for a typical experiment at an optimal PHA-concentration. With RPMI 1640 medium a relatively high thymidine incorporation was found in unstimulated cultures. When the medium was supplemented with lamb serum this value increased as well with MEMS as with RPMI medium. Higher PHA concentrations are needed for optimal transformation at 20 than at 10% horse serum.

With lymphocytes of all species replacement of horse serum by inactivated horse serum did not influence the thymidine incorporation. Also no large variations were observed with various batches of horse serum. With lymphocytes of pig a maximal thymidine incorporation was found at 2 days, with ovine and human lymphocytes at 2 or 3 days and with equine lymphocytes at 3 days. Values of {6-^{3}H}-thymidine incorporation at 51 hr in MEMS medium supplemented with horse serum are given in Table 6.2. Stimulation indices (ratio of radioactivity incorporated into PHA-stimulated lymphocytes to that in cultures without PHA) at 51 hr were higher than 37 for human, higher than 10 for equine, higher than 16 for porcine and higher than 10 for ovine lymphocytes. The optimal conditions for PHA-stimulation in MEMS medium are summarized for lymphocytes of the four species in Table 6.3.

Table 6.3. Optimal cultivation conditions for PHA-stimualtion of mammalian lymphocytes

Species	% horse serum	10^6 cells/ml	µg PHA/10^6 cells
Man	10	0.5 - 1	20
Horse	20	1 - 2	0.5 - 5.0
Pig	10	1 - 2	5.0
Sheep	10	1 - 2	10 - 40

Cultures are performed in MEMS medium with additions as described in Materials and Methods

6.4. Discussion

Measurement of incorporation of labelled thymidine is widely used as a parameter for PHA-stimulation of lymphocytes. The incorporation is dependent on the proportion of radioactive and unlabelled thymidine and its concentration and on the length of the incubation period. Our optimal thymidine concentration is comparable with those reported for Chinese hamster lymphocytes (81) and human lymphocytes (255).

Stimulation of human lymphocytes by PHA has extensively been reviewed and discussed (211). Lazary et al. (207) stimulated equine leucocytes with PHA in TC 199 medium and reported comparable optimal values for PHA and cell concentration. Ferrante & Thong (99) and Magnuson & Perryman (221) used comparable PHA concentrations for stimulation of purified equine lymphocytes in RPMI 1640 medium. Porcine lymphocytes respond very well to PHA, but horse serum and MEMS have not been used before. Optimal PHA and cell concentration are comparable to values reported previously (103, 193) in other media. Ovine lymphocytes responded slightly to PHA in MEMS and RPMI 1640 medium in the presence of lamb serum in contrast to in McCoy's 5A modified medium (55). The widely used RPMI 1640 medium gave moreover a high background stimulation. A similar optimal PHA concentration was reported by Fahey et al. (97), but the optimal PHA concentration reported by Chandra et al. (55) is 100 µg/10^6 cells. We found a similar dependence of the degree of transformation of porcine and ovine lymphocytes on the serum/PHA ratio as Forsdyke (103) for porcine and De Jong et al. (81) for hamster lymphocytes.

The conditions described in Table 6.3 for PHA-stimulation of mammalian lymphocytes give reproducible transformation rates and stimulation indices. The use of the same type of medium and serum

in studies of PHA-stimulation of lymphocytes of mammalian species may create better possibilities for investigation of the influence of various metabolites and antimetabolites on lymphocyte metabolism and function.

6.5. Summary

1. Optimal conditions for stimulation by PHA were established for equine, porcine, ovine and human lymphocytes in MEMS medium.
2. Optimal thymidine concentration was determined for assay of cell transformation. With all species tested horse serum gave the highest thymidine incorporation. Homologous serum was not more appropriate for lymphocytes of man, pig and sheep. 3. Optimal stimulation was achieved at 20, 0.5 - 5.0, 5, and 10 - 40 µg PHA per 10^6 cells for human, equine , porcine and ovine lymphocytes, respectively.

Chapter 7

EFFECTS OF ADENOSINE AND DEOXYADENOSINE ON PHA-STIMULATION OF LYMPHOCYTES OF MAN, HORSE AND PIG *

7.1. Introduction

Adenosine and deoxyadenosine can influence immune function. Deficiency of ADA in human lymphocytes has found to be associated with SCID (122). A higher activity of ADA has been found in some types of leukemic cells (22, 59, 70, 232). The concentrations of adenosine and deoxyadenosine were increased in erythrocytes, plasma and urine of patients with ADA deficiency (64, 157, 206). A higher concentration of dATP has been found in their erythrocytes and lymphocytes (64, 85, 157). Another adenosine-metabolizing enzyme SAH hydrolase is inactivated in erythrocytes of patients with ADA-deficiency (144). This may result in accumulation of SAH, that can inhibit methylation reactions (144, 188).

Several reports have been published on the effects of adenosine and deoxyadenosine on stimulation of human lymphocytes (13, 29, 106, 132, 152, 167, 334, 343, 386). Comparison of their results is difficult since various conditions were used. The kind, percentage of serum, kind of medium, kind of mitogen, the parameters tested and the time they were tested, vary. Moreover, inhibitors of ADA were used several times. These inhibitors potentiate the toxic effects of adenosine and deoxyadenosine (106, 152, 334), but they also influence cellular mechanisms to a large extent (136).

A very low activity af ADA as well with adenosine as with deoxyadenosine is present in lymphocytes of horse and pig (217, 368, chapter 5). This activity is comparable with the residual activity in some patients with SCID associated with ADA deficiency (155, 261). Mitogenic responsiveness of lymphocytes of these patients appears to be absent or markedly reduced (319, 386, 430) depending on the extent of residual ADA activity. Earlier we observed marked differences in activities and kinetics of adenosine and deoxyadenosine metabolizing enzymes between lymphocytes of man and those of horse and pig (chapter 5). Therefore we compared in this study the effects of

*adapted from Peters et al. (271)

adenosine and deoxyadenosine on PHA-stimulation of lymphocytes from man, horse and pig by assay of thymidine, uridine and leucine incorporation. To test some hypotheses on adenosine and deoxyadenosine toxicity to lymphocytes we also studied the effect of additional EHNA, an ADA inhibitor, homocysteine and deoxycytidine and measured the PRPP concentration in PHA-stimulated cells in the presence of adenosine. The species-related effects can partially be explained by the differences found in adenosine and deoxyadenosine metabolism (chapter 5).

7.2. Materials and methods

7.2.1. Materials

Ficoll (MW 400 kD) was obtained from Pharmacia and Isopaque from Nyegaard & Co., Oslo, Norway. A Ficoll-Isopaque solution was prepared as described previously (370). MEMS and Mycoplasma-screened horse serum were from Flow Laboratories, Irvine, UK. Bacto-Phytohem-agglutinin-P was a product from Difco Laboratories, Detroit, MI, USA. EHNA was a gift from Burroughs Wellcome Co., Research Triangle Park, NC, USA. {5-^{3}H}Uridine, {6-^{3}H}thymidine, L-{4,5-^{3}H}leucine were obtained from the Radiochemical Centre, Amersham, UK. All other chemicals were from the highest quality commercially available.

Blood samples were taken in heparinized bottles from healthy adult volunteers and adult horses (Equus caballus) and pigs (Sus scrofa).

7.2.2. Methods

Lymphocytes were isolated as described in chapter 5. Cells were cultured in a volume of 0.2 ml in U-shaped microtitration multi-well plates (Flow Laboratories, Irvine, UK) under conditions described in chapters 3 and 6. Horse serum was inactivated (30 min at 56°C) to eliminate ADA activity of the serum. Cultures were performed in at least quadruplicate. Leucine incorporation was measured at 43 hr and thymidine and uridine incorporation were measured both at 51 and 75 hr. 0.23 mM L-{4,5-^{3}H}leucine (57 mCi/mmol) was added after 27 hr cultivation and 2.6 µM {6-^{3}H}thymidine (3.3 Ci/mmol) or 2.6 µM {5-^{3}H}uridine (3.3 Ci/mmol) were present during the last 6 hr of incubation. The cells were isolated with a Titertek Cell Harvester (Flow Laboratories, Irvine, UK). Radioactivity was estimated as des-

cribed in chapters 3 and 6. When the effects of nucleosides or other compounds were investigated they were dissolved in medium, and first pipetted into the wells. PHA was added just before the cells were pipetted into the wells. The effect of additions is calculated as follows:

$$\frac{\text{culture with addition(s) (dpm in PHA-culture - dpm in culture without PHA)}}{\text{culture without addition(s) (dpm in PHA-culture - dpm in culture without PHA)}} \times 100\%$$

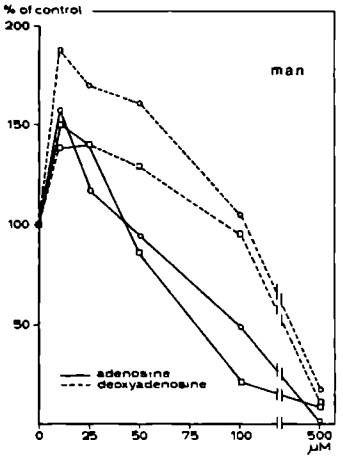
PRPP concentration in cultures was measured as described in chapter 3.

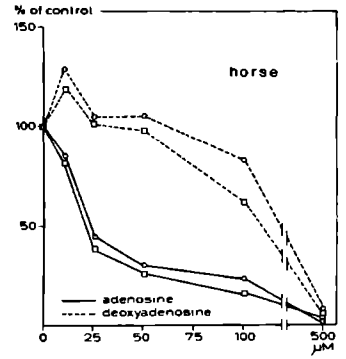
Reversed-phase HPLC was conducted on 0.4 M perchloric acid-extracted culture media using a Spectra-Physics SP 8700 solvent delivery system and a Merck LiChrosorb RP 18 (5 µm) column. Nucleosides and bases were detected at 254 nm. Elution (with a flow rate of 0.9 ml/min) started with 100% 0.04 M KH_2PO_4 (pH 6.5) during the first 13 min, followed by a linear gradient with increasing amounts of methanol and water (1:2, v/v). From 25 until 35 min the column was eluted with a mixture of 25% methanol, 50% water and 25% 0.04 M KH_2PO_4 (pH 6.5).

7.3. Results

7.3.1. Effects of adenosine and deoxyadenosine

Culture conditions were optimized for {6-^{3}H}thymidine incorporation, which is a parameter for DNA synthesis. Incorporation of {5-^{3}H}uridine and L-{4,5-^{3}H}leucine were used as parameters for RNA and protein synthesis, respectively. Nucleoside and leucine concentrations were optimized to minimize inhibition of their uptake and incorporation by other nucleosides or amino acids. In general, optimal thymidine incorporation was found with about similar values at 51 and 75 hr. Stimulation index (ratio of activity incorporated into PHA-stimulated cultures to that into control cultures) at 51 hr was higher than 37 for human, higher than 10 for equine and higher than 16 for porcine lymphocytes. For uridine incorporation these values were 15, 4 and 3, respectively, and for leucine incorporation 5, 4 and 4, respectively. Uridine incorporation reached an optimal value at 51 hr with all species. Leucine incorporation was measured at 43 hr, before uridine incorporation reached a optimum, to minimize the possibility that inhibition of protein synthesis would result from





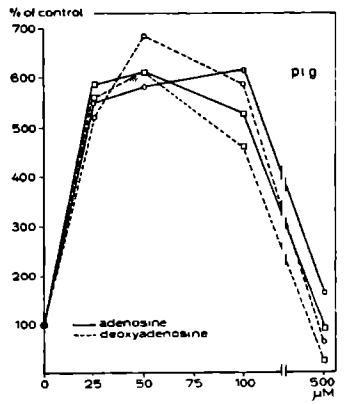


Fig. 7.1 - 7.3. Influence of adenosine and deoxyadenosine on thymidine (o) and uridine (□) incorporation of human, equine and porcine PHA-stimulated lymphocytes, respectively. Values are means of 3-4, 4-9 and 4-6 experiments, respectively and are calculated as described in section 7.2.2. Variation coefficient of the values did not exceed 30, 30 and 40%, respectively. Thymidine and uridine incorporation were measured at 51 hr.

For human control PHA-stimulated cultures thymidine incorporation ranged from 29270-118000 dpm and uridine incorporation from 51310 - 149720 dpm, and in cultures without PHA from 386 - 1333 dpm and from 2847 - 7378 dpm, respectively.

For equine control PHA-stimulated cultures thymidine incorporation ranged from 11790 - 65247 dpm and uridine incorporation from 36993 - 113788 dpm, and in cultures without PHA from 945 - 2767 dpm and from 6488 - 15345 dpm, respectively.

For porcine control PHA-stimulated cultures thymidine incorporation ranged from 69225 - 138976 dpm and uridine incorporation from 26565 - 123932 dpm, and in cultures without PHA from 1828 - 7202 dpm and from 8566 - 19554 dpm, respectively.

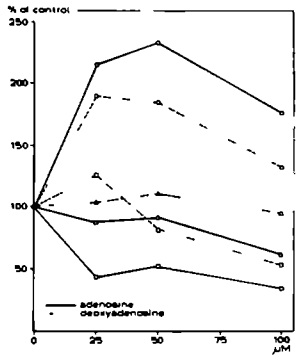


Fig. 7.4. Influence of adenosine and deoxyadenosine on leucine incorporation of human (Δ), equine (□) and porcine (o) PHA-stimulated lymphocytes. Values are means of 3-4 experiments and are calculated as described in section 7.2.2. Variation coefficient of all values did not exceed 30%. Leucine incorporation was measured at 43 hr and ranged in control PHA-stimulated cultures from 11980-63237 dpm for human lymphocytes, from 37270-58670 dpm for equine lymphocytes and from 19957-88330 dpm for porcine lymphocytes. The values for cultures without PHA were 2847-7378 dpm, 3236-15990 dpm and 1781-20298 dpm respectively.

inhibition of RNA synthesis in the experimental cultures with additions.

The effects of adenosine and deoxyadenosine on thymidine and uridine incorporation of human, equine and porcine lymphocytes at 51 hr are shown in Figs. 7.1 - 7.3, respectively. With all three species the effects on thymidine and uridine incorporation were comparable. Between the various species marked differences were found. The strongest inhibition with both nucleosides was found with equine lymphocytes. Adenosine already inhibited at a concentration of 10 µM. With human lymphocytes 50 µM adenosine was necessary for inhibition. Sensitivity to deoxyadenosine was lower than to adenosine. Deoxyadenosine stimulated at a low concentration in human and equine lymphocytes and inhibited only at a high concentration. With porcine lymphocytes both adenosine and deoxyadenosine stimulated thymidine and uridine incorporation 5- to 7-fold, even at 100 µM concentration. When thymidine incorporation was measured at 75 hr (Table 7.1), the effects of adenosine and deoxyadenosine were less pronounced. With equine lymphocytes adenosine showed a lower inhibition of thymidine incorporation while deoxyadenosine showed a comparable effect. When thymidine incorporation was compared in lymphocytes of the same pigs at 51 and 75 hr stimulation by adenosine and deoxyadenosine was lower after longer cultivation periods. Leucine incorporation was also affected by adenosine and deoxyadenosine (Fig. 7.4) but the degree of inhibition (with equine lymphocytes) or stimulation (with porcine lymphocytes) was lower than that of thymidine or uridine incorporation. Leucine incorporation in human lymphocytes was not significant-

Table 7.1. Influence of adenosine and deoxyadenosine on thymidine incorporation of PHA-stimulated lymphocytes after different incubation periods

Addition	Relative incorporation (in % of control)			
	Horse		Pig	
	51 hr	75 hr	51 hr	75 hr
Adenosine:				
500 µM	3 ± 3 (5)	3 ± 3 (6)	68 ± 37 (4)	N.D.
100 µM	16 ± 15 (5)	64 ± 31 (6)	532 ± 158 (5)	424 ± 237 (4)
50 µM	26 ± 6 (4)	57 ± 27 (5)	610 ± 139 (5)	432 ± 222 (4)
25 µM	39 ± 12 (9)	133 ± 33 (3)	584 ± 196 (6)	294 ± 56 (4)
10 µM	83 ± 28 (4)	125 ± 31 (4)	N.D.	N.D.
Deoxy-adenosine:				
500 µM	4 ± 2 (5)	0.7 ± 0.4 (3)	16 ± 13 (4)	N.D.
100 µM	61 ± 21 (4)	85 ± 40 (6)	459 ± 167 (5)	355 ± 155 (4)
50 µM	98 ± 12 (4)	111 ± 27 (5)	608 ± 158 (5)	484 ± 181 (4)
25 µM	102 ± 15 (8)	132 ± 22 (4)	548 ± 222 (6)	347 ± 161 (4)
10 µM	122 ± 24 (3)	113 ± 21 (6)	N.D.	N.D.

Values are means ± SD for the number of experiments indicated within parentheses and are calculated as described in section 7.2.2. N.D., not determined.

ly affected by deoxyadenosine.

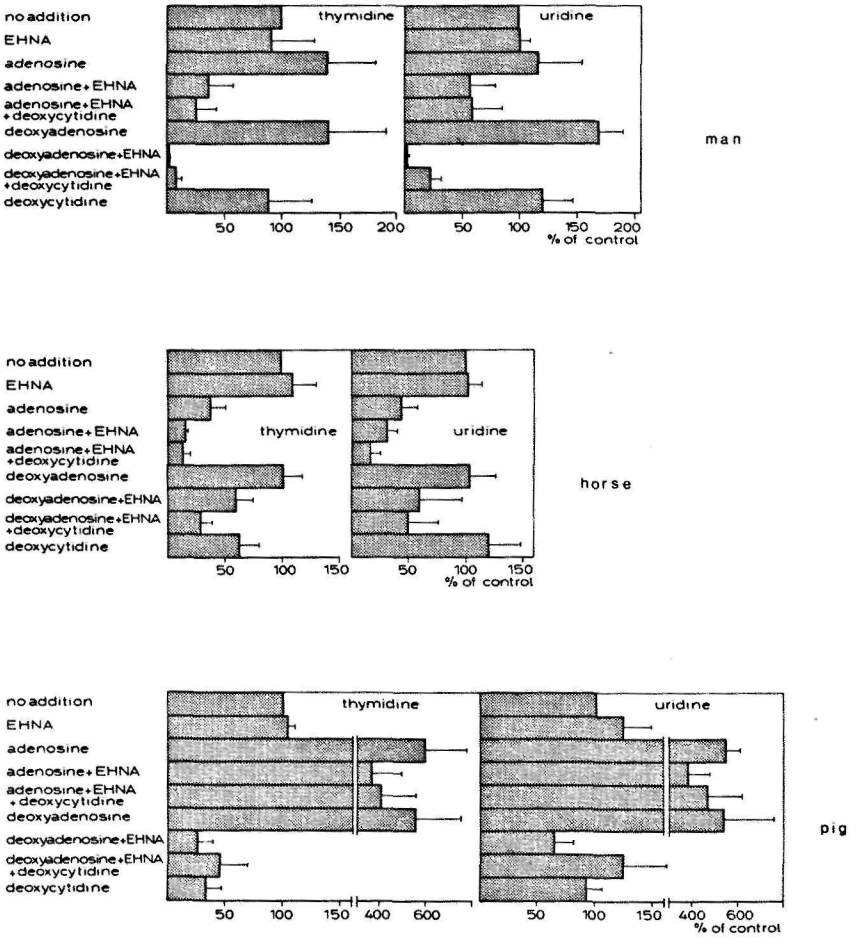
In the cultures adenosine and deoxyadenosine deamination were limited to cellular breakdown, since the deaminating activity of horse serum was reduced by inactivation from 8.6 to less than 2.5 nmol/hr per ml serum. Media in which human, equine and porcine lymphocytes had been stimulated by PHA in the presence of adenosine or deoxyadenosine were analyzed with reversed-phase HPLC. With all species a rapid disappearance (more than 70% within 24 hr) of adenosine or deoxyadenosine was found at 25-500 µM. Especially at high concentrations of both nucleosides the concentrations of (deoxy)inosine and hypoxanthine in the media increased. In the presence of EHNA a slower rate of disappearance of 25 µM (deoxy)adenosine from the medium was found (less than 30% within 24 hr). Hypoxanthine formation from deoxyadenosine was more suppressed by EHNA than its formation from adenosine.

7.3.2. The effects of EHNA and combinations of nucleosides

The effects of EHNA in combination with either adenosine or deoxyadenosine were investigated on human, equine and porcine lymphocytes (Figs. 7.5-7.7, respectively). EHNA alone did not significantly influence thymidine and uridine incorporation with all species but it changed the effects of adenosine and deoxyadenosine. With human lymphocytes especially with deoxyadenosine (Fig. 7.5) a markedly higher inhibition was observed in the presence of EHNA. With equine lymphocytes the effect of EHNA was lower with both nucleosides (Fig. 7.6), while with porcine lymphocytes EHNA slightly decreased the stimulatory effect of adenosine (Fig. 7.7). In contrast, EHNA reverted the stimulation of thymidine and uridine incorporation by deoxyadenosine into a marked inhibition.

Deoxycytidine has been reported to reverse inhibition by deoxyadenosine in human lymphocytes (29), but actually this effect should be called a prevention of inhibition (138). With human lymphocytes no prevention was found with deoxycytidine of suppression by adenosine plus EHNA, while inhibition by deoxyadenosine plus EHNA was lower in the presence of deoxycytidine. With equine lymphocytes deoxycytidine did not prevent inhibition by adenosine or deoxyadenosine in the presence of EHNA. With porcine lymphocytes deoxycytidine completely prevented the inhibition by deoxyadenosine plus EHNA of uridine incorporation and partially the inhibition of the thymidine incorporation. Deoxycytidine alone was tested at 25, 50 and 100 μM concentration. No concentration-dependent differences were found with all species tested but the effect on thymidine and uridine incorporation was different. In most cases thymidine incorporation was inhibited while uridine incorporation was stimulated or only slightly inhibited.

In vivo ADA deficiency causes accumulation of both adenosine and deoxyadenosine. Therefore we studied the effect of the combination of these nucleosides on thymidine incorporation of PHA-stimulated equine lymphocytes (Table 7.2). Adenosine and deoxyadenosine together did not show a stronger inhibition than adenosine alone. Addition of EHNA to the combination gave an inhibition comparable to that of deoxyadenosine and EHNA together. The effect of homocysteine was also studied in equine lymphocytes, since in these cells activity of SAH hydrolase is affected most strongly by deoxyadenosine (chapter 5). Homocysteine did not alter the inhibition found by adenosine or deoxyadenosine, but increased the inhibition by the combination.



Figs. 7.5 - 7.7. Influence of various nucleosides on thymidine and uridine incorporation on PHA-stimulated lymphocytes from man, horse and pig, respectively. Values are means ± SD of 3-6, 3-8 and 3-5 experiments, respectively, and are calculated as described in section 7.2.2. Thymidine and uridine incorporation were measured at 51 hr. Concentrations of adenosine, deoxyadenosine and deoxycytidine were 25 µM. Concentration of EHNA was 10 µM.

Table 7.2. Influence of various combinations on thymidine incorporation of PHA-stimulated equine lymphocytes

Addition \ Combination	None	+ EHNA	+ Homocysteine	+ EHNA + homocysteine
None	100	110 ± 20 (4)	54 ± 18 (3)	N.D.
Adenosine	39 ± 12 (9)	18 ± 7 (8)	46 ± 14 (4)	17 ± 4 (3)
Deoxyadenosine	102 ± 15 (8)	50 ± 15 (5)	87 ± 18 (4)	33 ± 10 (4)
Adenosine + deoxyadenosine	32 ± 7 (3)	14 ± 1 (3)	21 ± 2 (3)	13 ± 4 (3)

Values (in % of the control without additions) are means ± SD for the number of experiments indicated within parentheses and are calculated as described in section 7.2.2. Adenosine and deoxyadenosine were present in 25 µM, homocysteine in 100 µM and EHNA in 10 µM final concentration. Thymidine incorporation was measured at 51 hr. N.D., not determined.

Additional EHNA only increased the effect of deoxyadenosine plus homocysteine (Table 7.2).

To get more insight about the mechanism by which adenosine stimulates thymidine and uridine incorporation of porcine lymphocytes, the effects of the degradation products of adenosine on thymidine and uridine incorporation were studied (Table 7.3). Adenosine, inosine and hypoxanthine showed a comparable stimulation of thymidine and uridine incorporation, especially when compared within the same experiment. With human lymphocytes 100 µM inosine did not influence thymidine and uridine incorporation in contrast to adenosine (see also Fig. 8.2).

7.3.3. The effect of adenosine on the PRPP concentration

We also measured the effect of adenosine on PRPP concentration of PHA-stimulated lymphocytes. As previously reported (chapter 3) the PRPP concentration increases in PHA-stimulated equine and porcine lymphocytes. Adenosine suppresses this elevation during cultivation of equine lymphocytes and PRPP concentration decreases to below the value at zero hr (Table 7.4). With porcine lymphocytes the suppression of the PHA-stimulated elevation was more marked, but the concentration remained somewhat higher than that at zero hr. Without PHA-stimulation adenosine inhibited the increase of PRPP concentration after three hr.

Table 7.3. Influence of adenosine, inosine and hypoxanthine on thymidine and uridine incorporation of PHA-stimulated porcine lymphocytes

Addition	Concentration (in µM)	Relative incorporation (in % of the control value)	
		Thymidine	Uridine
Adenosine	100	459 ± 167 (5)	590 ± 202 (4)
	50	490 ± 198 (7)	471 ± 210 (6)
Inosine	100	345 ± 69 (3)	462 ± 214 (6)
	50	608 ± 158 (5)	684 ± 316 (4)
Hypoxanthine	100	490 ± 211 (7)	334 ± 123 (3)
	50	319 ± 63 (3)	359 ± 117 (3)

Values are means ± SD for the number of experiments indicated within parentheses and are calculated as described in section 7.2.2. Incorporation was measured at 51 hr.

7.4. Discussion

Information on effects of adenosine and deoxyadenosine on human lymphocyte function is scattered. Most authors only studied the effects of either adenosine or deoxyadenosine. ADA inhibitors were often used without studying the effect of the inhibitor or the nucleoside alone. Although Hirschhorn & Sela (152) and Magnuson & Perryman (221) demonstrated that inhibition by adenosine of thymidine incorporation of human PHA-stimulated lymphocytes decreases at longer incubation periods, most results are based on thymidine and leucine incorporation measured at 72 hr. We showed that the effects of both adenosine and deoxyadenosine are higher at shorter incubation times with equine and porcine lymphocytes. This means that during the longer incubation period the cells will eliminate the excess amount of nucleoside, recover and start again normal thymidine incorporation. We found comparable, high elimination rates of adenosine from the culture medium for lymphocytes of all three species, as reported by Uberti et al. (386, 387) and Hirschhorn & Sela (152) for human lymphocyte cultures. Our results on the effect of adenosine on the thymidine incorporation of human lymphocytes at 48 hr agree with those found previously (132, 152, 167, 231). Magnuson & Perryman (231) reported comparable effects of adenosine on PHA-stimulated equine lymphocytes as we found. In human lymphocytes Simmonds et al. (334) found about 40% inhibition at 100 µM deoxyadenosine. No data are a-

Table 7.4. Effect of adenosine and PHA on the relative PRPP concentration in cultured lymphocytes of horse and pig

Species	Culture time (hr)	Without PHA		With PHA	
		-Adenosine	+Adenosine	-Adenosine	+Adenosine
Horse	3	189 ± 31	37 ± 14	239 ± 51	35 ± 21
	24	101 ± 52	56 ± 15	218 ± 38	60 ± 11
	48	79 ± 43	48 ± 21	176 ± 29	91 ± 15
	72	75 ± 29	41 ± 10	109 ± 28	55 ± 13
Pig	3	681 ± 287	117 ± 74	919 ± 243	157 ± 86
	24	58 ± 36	54 ± 45	522 ± 288	81 ± 5
	48	115 ± 53	88 ± 17	1216 ± 391	154 ± 54
	72	53 ± 26	61 ± 39	1227 ± 105	136 ± 124

Values (in % of the concentration at the start of the cultures) are means ± SD of 3 experiments. Adenosine (50 µM final concentration) was added at the start of the cultures. Concentration of PRPP at 0 hr was 5.4 ± 0.2 and 4.1 ± 2.1 nmol/10^9 cells for equine and porcine lymphocytes, respectively.

vailable on the effects of deoxyadenosine on thymidine incorporation of equine and porcine lymphocytes.

Since {5-^{3}H}uridine is specifically incorporated into RNA, our results indicate that not only DNA synthesis but also RNA synthesis is influenced by adenosine and deoxyadenosine. The observed inhibition of leucine incorporation is not a secondary effect of inhibition of RNA synthesis, since leucine incorporation was measured before RNA synthesis starts to increase. Uberti et al. (387), Hirschhorn & Sela (152) and Carson et al. (46) found comparable effects of adenosine and deoxyadenosine on leucine incorporation of PHA-stimulated human lymphocytes as we did.

Adenosine appears to be more toxic than deoxyadenosine to human and equine lymphocytes. Lymphocytes of ADA-deficient patients, however, were more sensitive to deoxyadenosine (156). Addition of EHNA results in a higher toxicity of deoxyadenosine (334 and Fig. 7.4). Even with porcine lymphocytes both thymidine and uridine incorporation are inhibited in the presence of EHNA and deoxyadenosine. Other inhibitors of ADA, coformycin and deoxycoformycin, also potentiate the effect of adenosine and deoxyadenosine on thymidine and leucine incorporation of human lymphocytes (29, 343, 387). In the presence

of these inhibitors deoxyadenosine also is more toxic than adenosine. Apparently inhibition of deamination results in the synthesis of compounds that are more toxic when they are derived from deoxyadenosine than from adenosine. The highest inhibition by deoxyadenosine and EHNA was found with human lymphocytes. They can relatively better phosphorylate deoxyadenosine at low concentration than porcine and equine lymphocytes (chapter 5).

No significant differences on ATP and dATP concentrations were observed after adenosine addition with and without EHNA in lymphocytes of man, horse and pig. dATP, however, was present in 3-10 fold higher concentration in lymphocytes of all three species 24 hr after addition of deoxyadenosine, EHNA and PHA (chapter 9). dATP is an inhibitor of ribonucleotide reductase (301). Accumulation of dATP will affect cell growth and cell function (138, 281). Our results support the hypothesis that inhibition of lymphocyte function in ADA deficiency could be mediated by dATP accumulation.

Deoxycytidine, a precursor of dCTP, has been reported to be able to prevent deoxyadenosine toxicity (29, 46). This does not hold for lymphocytes of all species studied. The marked prevention of the inbitory effect of deoxyadenosine and EHNA by deoxycytidine in human lymphocytes and the absence of any preventive effect in equine lymphocytes agree well with the high inhibition of deoxyadenosine phosphorylation by deoxycytidine in human lymphocytes and the low inhibition in equine lymphocytes (chapter 5).

Although adenosine and deoxyadenosine are both present in elevated amounts in serum, plasma and urine of patients with ADA deficiency associated with SCID (85, 206), the effect of the combination of these nucleosides on lymphocyte function has not been studied before. In equine lymphocytes the combination had no stronger inbitory effect on PHA-stimualtion than adenosine alone. Addition of homocysteine to the combination caused a stronger inhibition of thymidine incorporation of PHA-stimulated equine lymphocytes. Since addition of homocysteine to either adenosine or deoxyadenosine had no effect, both adenosine and deoxyadenosine are necessary to cause an accumulation of SAH. This product, which was present in increased amounts in human lymphocytes after incubation with adenosine and homocysteine-thiolactone (188), can inhibit methylation reactions (188, 202).

In most cell types adenosine and deoxyadenosine are inhibitory to growth and/or toxic (111, 241, 391 and reviewed in 138). Although porcine lymphocytes have a relatively low ADA activity, no inhibition

but a marked stimulation of thymidine and uridine incorporation was observed, even at high concentrations of adenosine and deoxyadenosine. This stimulation can be mediated by delivery of hypoxanthine, since adenosine, deoxyadenosine, inosine and hypoxanthine showed comparable stimulation of thymidine and uridine incorporation. Hypoxanthine can be converted with PRPP by HPRT to IMP and subsequently to AMP and/or GMP. The suppression of the PHA-stimulated increase of PRPP concentration in the presence of adenosine may be related to this phenomenon, but also to inhibition of PRPP synthesis by AMP, a potent inhibitor of PRPP synthetase (chapter 3). It does not appear probably that depletion of the intracellular pyrimidine nucleotide pool causes the increased incorporation of labelled thymidine and uridine in the presence of adenosine or deoxyadenosine, since optimal concentrations of the pyrimidine nucleosides were present in the culture medium and uridine kinase activity is increased at PHA-stimulation in human (214), porcine and equine lymphocytes (chapter 4). With equine lymphocytes the decrease of the PRPP concentration by adenosine, even to below the level at zero hr, can only be due to inhibition of PRPP synthesis by AMP. Inhibition of the PHA-stimulated rise in PRPP concentration by adenosine was earlier found in human lymphocytes (343). In lymphoblasts adenosine reduced PRPP concentration (344); addition of coformycin further depleted the PRPP pool and also inhibited the hexose monophosphate shunt.

Our results indicate that the effects of adenosine and deoxyadenosine on human lymphocyte function may be related to various mechanisms. In the absence of ADA-inhibitors, adenosine is more inhibitory than deoxyadenosine, but inhibition by adenosine is not accompanied, even in the presence of ADA inhibitors, with an accumulation of ATP or dATP (chapter 9). The effects of adenosine may be mediated by inhibition of PRPP synthesis and accumulation of SAH, although inhibition of pyrimidine nucleotide synthesis (110, 125, 177) and accumulation of cAMP (226, 323, 431) may also contribute to the observed toxic phenomena in various types of cells. The effects of deoxyadenosine appear to be mediated by accumulation of dATP and SAH. However, little attention has been paid to other possible effects of deoxyadenosine like interference with pyrimidine metabolism, with synthesis of cAMP and PRPP and with energy metabolism. Schmalstieg et al. (318) found a decreased ATP/ADP ratio in lymphocytes of an ADA-deficient patient. Some of these aspects, especially those concerning pyrimidine metabolism and energy metabolism are currently under investigation.

7.5. Summary

1. Adenosine inhibits thymidine and uridine incorporation of PHA-stimulated lymphocytes of man and horse at concentrations higher than 50 and 10 μM, respectively. Deoxyadenosine is inhibitory at concentrations higher than 100 μM. Thymidine and uridine incorporation of porcine lymphocytes are elevated 5-7 fold by 25-100 μM adenosine, deoxyadenosine, inosine and hypoxanthine. Leucine incorporation of PHA-stimulated lymphocytes was affected by adenosine and deoxyadenosine in the same way, but to a lower extent. 2. Effects of adenosine and deoxyadenosine were more pronounced at shorter cultivation times. 3. EHNA potentiated the effects of adenosine and deoxyadenosine on human and equine lymphocytes. With human lymphocytes inhibition by deoxyadenosine and EHNA was higher than by adenosine and EHNA. With porcine lymphocytes only the combination of deoxyadenosine and EHNA was inhibitory. 4. Homocysteine potentiated the inhibition of thymidine incorporation by the combination of adenosine and deoxyadenosine with equine lymphocytes, but not the inhibition of adenosine or deoxyadenosine alone. 5. Adenosine suppressed the PHA-stimulated elevation of PRPP concentrations. With porcine lymphocytes PRPP remained at the level of zero hr, while with equine lymphocytes PRPP concentration decreased to below that level. 6. The various effects of adenosine and deoxyadenosine on lymphocytes of man, horse and pig can partially be related to differences in adenosine and deoxyadenosine metabolism.

Chapter 8

METABOLISM OF PURINE NUCLEOSIDES IN HUMAN AND OVINE LYMPHOCYTES AND RAT THYMOCYTES AND THEIR INFLUENCE ON MITOGENIC STIMULATION*

8.1. Introduction

Inherited deficiency of PNP in human lymphocytes is associated with severe T-cell dysfunction (123, 332). PNP catalyzes the phosphorylysis of inosine, guanosine and their deoxycompounds to hypoxanthine and guanine. The substrates of PNP appear to play a key role in the expression of the disturbed immune function (237, 281) and are found in increased amounts in erythrocytes, serum and urine of PNP deficient patients (63, 65, 302, 351, 352, 420). Furthermore an elevated concentration of dGTP has been found in erythrocytes of these patients (65, 352, 420). The activity of SAH hydrolase in their erythrocytes was also decreased (146, 190), probably by inactivation by inosine (146). It is postulated that the development and differentiation of T-cell mediated immunity in lymphoid tissues is mainly inhibited by the accumulated deoxynucleotides that inhibit ribonucleotide reductase and cause a depletion of pyrimidine dNTP (237, 281).

Deoxyguanosine appeared to be more toxic to T-cell derived cell lines (120, 235, 254) and T-lymphocytes (314) than to B-cell lines. Human thymocytes are more sensitive to deoxyguanosine than peripheral lymphocytes (67). However, it was also shown that dGTP accumulation is high in non-dividing lymphoid cells after a short incubation with deoxyguanosine (195).

Deoxyguanosine appears to be phosphorylated in lymphoid cells by deoxycytidine kinase (46, 216) and by a specific deoxyguanosine kinase (216). A high deoxyguanosine-phosphorylating activity was found in thymus, spleen and peripheral lymphocytes (46, 49, 216, 253, 254, 314). The kinetic properties of PNP and deoxyguanosine kinase and the metabolism of the substrates of PNP were not yet studied in lymphocytes.

Ovine lymphocytes have a very low PNP activity (chapter 5) that

*adapted from Peters et al. (274)

approximates the activity in children with PNP deficiency (315). Rat thymocytes have a comparably low PNP activity that also is lower than in human thymocytes (chapter 11). Knowledge of the metabolism of the substrates of PNP in these cells might give some insight in the role of PNP in immune function. Therefore we studied kinetics of phosphorylation and phosphorylysis of inosine, guanosine and deoxyguanosine in intact cells and extracts of human and ovine lymphocytes and rat thymocytes. The influence of inosine, guanosine and their deoxycompounds on mitogenic stimulation of these cells was also studied. The various effects of the nucleosides on this stimulation could partially be explained by the differences in their metabolism.

8.2. Materials and methods

8.2.1. Materials

Origin of most materials is described in chapters 5 and 6. Deoxy{8-^{3}H}guanosine and {8-^{3}H}guanosine were purchased from the Radiochemical Centre, Amersham, UK. Concanavalin-A (from *Canavalia ensiformis*) from Boehringer, Mannheim, FRG.

Blood samples were taken in heparinized bottles from healthy adult volunteers and Texel sheep (*Ovis aries*). Thymocytes were prepared from thymuses of 40-days old male Wistar rats.

8.2.2. Enzyme assays

Isolation of lymphocytes and preparation of extracts and supernatants is described in chapter 5. Thymocytes were purified as described in chapter 10.

All enzyme activities were measured by radiochemical methods at 37°C in a shaking water-bath. Activities are expressed as nmol of product(s) formed per hr per 10^6 cells. Proportionality of the reactions with respect to time and the amount of protein was ascertained. Reactions were terminated by heating for 2-5 min at 95°C in an Eppendorf heater.

The reaction mixture (50 µl) for the assay of PNP activity contained 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 80 mM phosphate (pH 7.4) and an amount of lymphocyte or thymocyte lysate equivalent to $3 - 25 \times 10^4$ cells. The reaction was started after a preincubation of 5 min by addition of substrate. Final concentrations were 0.34

mM {8-^{14}C}inosine (7.3 mCi/mmol), 0.29 mM {8-^{3}H}guanosine (30 mCi/mmol) or 0.29 mM deoxy{8-^{3}H}guanosine (30mCi/mmol), unless otherwise indicated. After 10 min the reaction was terminated and inosine and hypoxanthine, or guanosine or deoxyguanosine and guanine and xanthine, were added as carriers.

The reaction mixture (80 µl) for measuring phosphorylation of guanosine or deoxyguanosine contained 40 mM Tris-HCl (pH 7.4), 0.625 mM EDTA, 5 mM ATP, 6.25 mM $MgCl_2$, a volume of 7000 g supernatant equivalent to 2 - 3 x 10^6 cells and 0.33 mM {8-^{3}H}guanosine (134 mCi/mmol) or 0.33 mM deoxy{8-^{3}H}guanosine (134 mCi/mmol). After incubation for 60-90 min the reaction was terminated by heating and addition of excess GMP and guanosine or their deoxycompounds and guanine and xanthine. Substrates and products (nucleotides and bases) were separated on PEI-cellulose thin-layers with distilled water and quantitated as descibed in chapter 5.

8.2.3. Incubations with intact cells

To determine the rate of phosphorylation and phosphorylysis in intact cells, 4 x 10^6 cells were suspended in 100 µl 20 mM Hepes-buffered (pH 7.0) MEMS-medium. {8-^{14}C}Hypoxanthine, {8-^{14}C}inosine, {8-^{3}H}guanosine or deoxy{8-^{3}H}guanosine was added at concentrations indicated in Figures and Tables. After 60 min the incubation was terminated by spinning down the cells at 10 000g for 1 min. The cells were washed with 100 µl saline. The medium was added to 65 µl of a solution containing appropriate carriers and combined with the washing fluid. The cells were extracted with 100 µl 0.4 M $HClO_4$ on ice. After 20 min the extract was centrifuged at 10 000 g for 1 min and the supernatant neutralized with 29 µl of a mixture of 0.4 M KOH and 1 M K_2HPO_4. After centrifugation the supernatant was combined with the medium and the washing fluid, and analyzed on PEI-cellulose thin-layers. 0.2 ml of 1 M hydroxide of Hyamine 10-X was added to the acid-insoluble material and left overnight at room temperature. Radioactivity was measured in 10 ml of toluene containing 0.4 g dimethyl-POPOP and 6 g PPO per 1. Blancs were obtained by spinning down the cells immediately after addition of the substrate.

8.2.4. Cell cultures

Lymphocytes were cultured as described in chapter 6. Horse serum was inactivated (30 min at 56°C) to reduce its PNP activity.

The PNP activity (with inosine as substrate) decreased from 101 to 28 nmol of hypoxanthine formed per hr per ml serum. Rat thymocytes (2×10^6 cells per ml) were cultured in microtitertek plates in 22 mM bicarbonate-buffered RPMI-1640 medium supplemented with 10% inactivated horse serum, 2 mM L-glutamine and 100 µg streptomycin and 100 Units penicillin per ml. The thymocytes were stimulated with 2.5 µg Con-A per 10^6 cells. For comparison human lymphocytes were also cultured in RPMI-1640 medium supplemented with 10% inactivated horse serum in a cell concentration of 0.5×10^6 cells/ml and stimulated with 15 µg Con-A per 10^6 cells. All cultures (0.2 ml) were performed under 95% air/5% CO_2 in at least quadruplicate. Thymidine and uridine incorporation were measured at 51 hr; 2.6 µM {6-^{3}H}thymidine (3.3 Ci/mmol) or 2.6 µM {6-^{3}H}uridine (3.3 Ci/mmol) was present during the last 6 hr of incubation. Quantitation of incorporated radioactivity was performed as described in chapter 6. Determination and calculation of the effects of added nucleosides is described in section 7.2.2.

8.3. Results

8.3.1. PNP and nucleoside kinase(s)

In PNP assays with inosine as a substrate the only product was hypoxanthine, with deoxyguanosine and guanosine both guanine and xanthine were formed. With human lymphocytes the PNP activity was highest with inosine as a substrate, while the Km values for all substrates were comparable (Table 8.1). Ovine lymphocytes showed

Table 8.1. Activities and Km values of PNP in lysates of human and ovine lymphocytes and rat thymocytes

Species	Parameter	Inosine	Guanosine	Deoxyguanosine
Man	Activity	248 ± 131 (6)	176 ± 48 (6)	128 ± 26 (4)
	Km	93 ± 28 (5)	134 ± 48 (6)	105 ± 28 (4)
Sheep	Activity	9 ± 4 (8)	12 ± 5 (8)	17 ± 5 (7)
	Km	54 ± 3 (3)	49 ± 11 (5)	99 ± 17 (5)
Rat	Activity	26 ± 6 (7)	N.D.	13 ± 5 (4)
	Km	N.D.	N.D.	98 ± 23 (4)

Activities (in nmol/hr per 10^6 cells) and Km values (in µM) are means ± SD for the number of individuals given in parentheses. N.D., not determined.

much lower but comparable activities with the three substrates, but the Km value for deoxyguanosine was much higher than that for the other nucleosides. With rat thymocytes activity of PNP with inosine and deoxyguanosine was comparable. Km values for deoxyguanosine were comparable with all cell types.

The assay for kinase activity was optimized for deoxyguanosine with extracts of thymocytes. At 0.33 mM deoxyguanosine the combination of 5 mM ATP, 6.25 mM $MgCl_2$ and 625 µM EDTA appeared to be optimal. No difference in activity was found when the cells were lysed by sonication or by freezing and thawing. Addition of dithioerythritol, dithiothreitol or of BSA (5%, w/v) did not influence the enzyme activity. Since no difference in activity was found between a sonified total lysate and its 7 000 g supernatant, the deoxyguanosine-phosphorylating activity will be located in the cytosol. Therefore all activities were measured in 7 000 g supernatants.

The highest deoxyguanosine kinase activity and the highest Km value were found with human lymphocytes (Table 8.2). At 0.33 mM the phosphorylation rate of deoxyguanosine in extracts of ovine lymphocytes was comparable with that of guanosine. The deoxyguanosine kinase activity per mg protein at 0.33 mM was about 21 nmol per hr with extracts of human lymphocytes and about 7 nmol per hr with extracts of ovine lymphocytes and rat thymocytes. With extracts of ovine lymphocytes 0.5 mM guanine did not inhibit conversion of guanosine and deoxyguanosine to nucleotides. In extracts of rat thymocytes no guanosine phosphorylating activity was detectable.

Table 8.2. Activities and Km values of nucleoside kinase(s) for guanosine and deoxyguanosine in 7 000 g supernatants from human and ovine lymphocytes and rat thymocytes

Species	Deoxyguanosine			Guanosine
	Km	Vmax	Activity (at 0.33 mM)	Activity (at 0.33 mM)
Man	1565 ± 670 (3)	4.5 ± 1.8 (3)	0.68 ± 0.13 (4)	0.14 ± 0.05 (4)
Sheep	101 ± 31 (3)	0.22 ± 0.10 (3)	0.19 ± 0.08 (4)	0.17 ± 0.05 (4)
Rat	789 ± 354 (3)	0.18 ± 0.09 (3)	0.10 ± 0.05 (8)	<0.02

Activities (in nmol/hr per 10^6 cells) and Km values (in µM) are means ± SD for the number of individuals given in parentheses.

8.3.2. Metabolism of nucleosides by intact cells

With intact cells the rate of phosphorylysis (Table 8.3) is only a few percent of that in extracts. Furthermore the differences in phosphorylysis rates between the various cell types are less pronounced with the intact cells. With intact rat thymocytes about 40% of guanine formed from guanosine or deoxyguanosine was deaminated to xanthine. The rate of phosphorylation by intact cells includes the incorporation of nucleoside (or base) into nucleotides and nucleic acids (acid-insoluble material). The rate of phosphorylation of deoxyguanosine and guanosine found with intact cells was comparable in most cases with that found with extracts. With intact ovine lymphocytes the rate of phosphorylation was comparable with the rate

Table 8.3. Metabolism of deoxyguanosine, guanosine and inosine in intact human and ovine lymphocytes and rat thymocytes and the effect of hypoxanthine

Species	Substrate	Phosphorylysis rate		Phosphorylation rate	
Man	Deoxyguanosine	1.96 ± 0.50	(88 ± 17)	0.134 ± 0.045	(39 ± 20)
	Guanosine	1.58 ± 0.75	(106 ± 7)	0.145 ± 0.092	(45 ± 19)
	Inosine	2.68 ± 0.57	(64 ± 20)	0.069 ± 0.026	(7 ± 1)
Sheep	Deoxyguanosine	0.54 ± 0.29	(88 ± 18)	0.178 ± 0.052	(39 ± 13)
	Guanosine	0.69 ± 0.25	(86 ± 19)	0.235 ± 0.095	(56 ± 26)
	Inosine	0.88 ± 0.15	(34 ± 4)	0.107 ± 0.031	(7 ± 3)
Rat	Deoxyguanosine	0.52 ± 0.24	(104 ± 45)	0.098 ± 0.070	(54 ± 31)
	Guanosine	0.95 ± 0.18	(74 ± 23)	0.021 ± 0.007	(64 ± 38)
	Inosine	1.49 ± 0.13	(36 ± 6)	0.056 ± 0.012	(7 ± 1)

Rates (in nmol of product(s) formed per hr per 10^6 cells) are means ± SD for 3-6 experiments. The products of phosphorylysis are guanine and xanthine or hypoxanthine. Phosphorylation products include nucleotides and the acid-insoluble material. The values in the presence of 0.5 mM hypoxanthine are given between parentheses in % of the control activity. Concentration of guanosine and deoxyguanosine was 245 µM and that of inosine 142 µM.

of phosphorylysis at low deoxyguanosine concentration (Fig. 8.1A). With intact human lymphocytes and rat thymocytes phosphorylysis of deoxyguanosine was higher than phosphorylation at all concentrations (Fig. 8.1B & C). Guanosine and inosine were also incorporated into

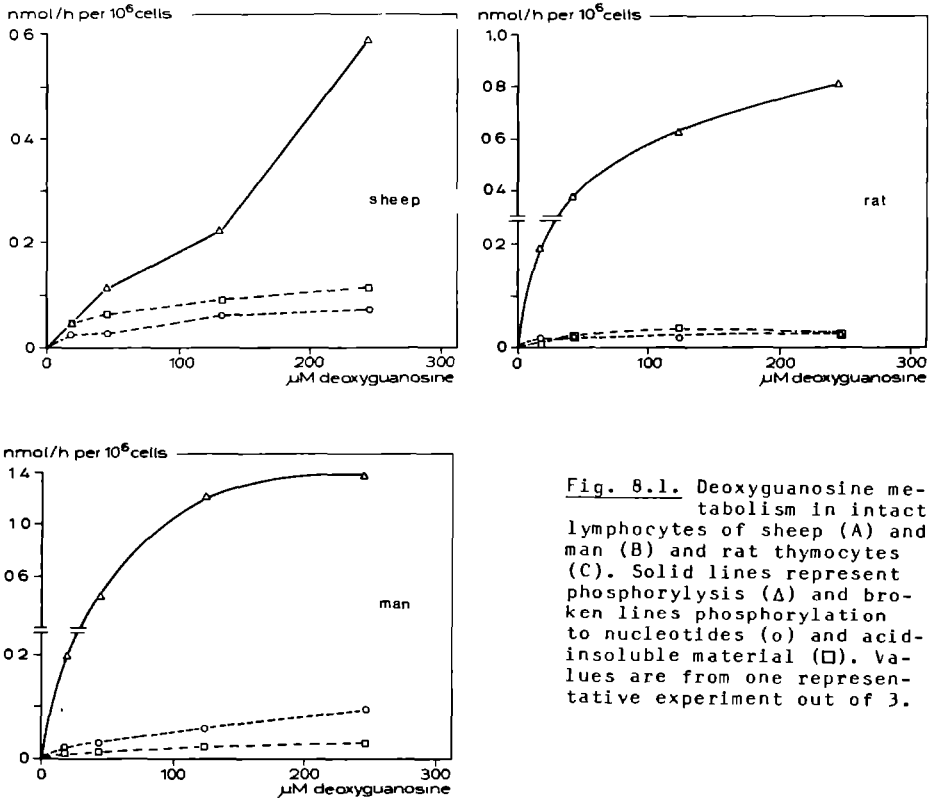


Fig. 8.1. Deoxyguanosine metabolism in intact lymphocytes of sheep (A) and man (B) and rat thymocytes (C). Solid lines represent phosphorylysis (Δ) and broken lines phosphorylation to nucleotides (o) and acid-insoluble material (□). Values are from one representative experiment out of 3.

nucleotides and nucleic acids of intact cells (Table 8.3), although the rate of phosphorylysis was much higher.

Phosphorylation of deoxyguanosine, guanosine and inosine in intact cells could be mediated by phosphorylysis and subsequent conversion of the base by HGPRT. This enzyme has a marked activity in human and ovine lymphocytes (chapter 2). Hypoxanthine and guanine are comparable substrates and do not only compete for the same site on the enzyme, but also for the second substrate PRPP. Hypoxanthine and guanine formed from inosine and deoxyguanosine or guanosine can reach a maximal concentration of 100 µM (with intact human lymphocytes) at the end of the incubation. We added 0.5 mM non-radioactive hypoxanthine or guanine to the reaction mixture to suppress possible conversion of the radioactive bases by HGPRT. Incorporation of [8-^{14}C]hypoxanthine (0.5 mM) into total nucleotides and nucleic

acids amounted to about 120, 78 and 40 pmol per hr per 10^6 cells for human and ovine lymphocytes and rat thymocytes, respectively. Hypoxanthine did not inhibit phosphorylysis of guanosine and deoxyguanosine, but phosphorylysis of inosine was significantly inhibited (Table 8.3). After addition of hypoxanthine no incorporation of the base unit of deoxyguanosine, guanosine or inosine into nucleotides was found. The incorporation of guanosine and deoxyguanosine into acid-insoluble material was considerably reduced, and that of inosine almost completely inhibited. With intact ovine lymphocytes 0.5 mM guanine inhibited by about 50% incorporation of deoxyguanosine and guanosine into nucleotides and nucleic acids. This indicates that at least a part of the found phosphorylation of guanosine and deoxyguanosine with intact cells is due to the route of PNP and HGPRT. This route probably completely accounts for the inosine incorporation. No inosine kinase appears to be present.

8.3.3. Influence of (deoxy)nucleosides on mitogenic stimulation

Culture conditions optimized for (6-^{3}H)thymidine incorporation were used to study the effects of (deoxy)nucleosides on PHA-stimulation of human and ovine lymphocytes. Inactivated horse serum was used in all cultures since it showed the lowest PNP activity, even lower than inactivated lamb serum. With rat thymocytes no appropriate stimulation index (ratio of radioactivity incorporated into cultures with mitogen to that in cultures without mitogen) could be obtained with PHA either in MEMS or RPMI 1640 medium. Since best results were obtained with Con-A stimulation in RPMI 1640 medium, these conditions were used to study the effects of (deoxy)nucleosides on rat thymocytes. Stimulation index for human lymphocytes was higher than 31 with thymidine and higher than 10 with uridine. For ovine lymphocytes stimulation index was higher than 10 and for rat thymocytes higher than 30 with thymidine.

Inosine and deoxyinosine had a comparable effect on thymidine and uridine incorporation of PHA-stimulated lymphocytes of man (Fig. 8.2). With human and ovine lymphocytes 500 µM deoxyinosine caused about 50% inhibition of incorporation in contrast to inosine. Guanosine (500 µM) and deoxyguanosine inhibited thymidine incorporation of PHA-stimulated human lymphocytes more than uridine incorporation (Fig. 8.3). At lower concentrations no significant inhibition was observed. Inhibition at 500 µM guanosine and deoxyguanosine was higher with human (Fig 8.3) than with ovine lymphocytes (Fig. 8.4).

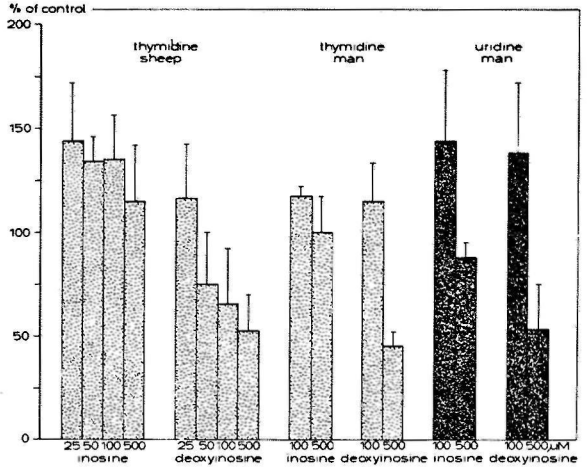


Fig. 8.2. Influence of inosine and deoxyinosine on thymidine and uridine incorporation of PHA-stimulated human lymphocytes and on thymidine incorporation of PHA-stimulated ovine lymphocytes. Values are means ± SD of 3-5 experiments. Thymidine and uridine incorporation were measured at 51 hr. Thymidine incorporation of control PHA-stimulated cultures of ovine and human lymphocytes ranged from 23 037-130 780 dpm and from 29 269-168 103 dpm, respectively, and in cultures without PHA from 2 340-4 161 dpm and from 386-1489 dpm , respectively. Uridine incorporation of control PHA-stimulated human lymphocytes ranged from 34 022-149 715 dpm and in cultures without PHA from 1479-4112 dpm.

The effects of inosine, guanosine and their deoxycompounds on human lymphocytes stimulated with Con-A in RPMI-1640 medium were comparable with those on PHA-stimulated cells. With rat thymocytes no inhibition of Con-A stimulated thymidine incorporation was found at 50 µM inosine and deoxyinosine and at 50 and 500 µM guanosine (Fig. 8.5). Thymidine incorporation was moderately inhibited at 500 µM inosine and deoxyinosine and at 50 µM deoxyguanosine, but it was completely inhibited at 500 µM deoxyguanosine.

8.4. Discussion

Activities of PNP in mammalian lymphocytes with inosine as a substrate are discussed in chapter 5. No simultaneous measurements of activities and Km values with inosine, guanosine and deoxyguanosine have been reported for lymphocytes. The activity with guanosine as substrate in human lymphocytes reported by North et al. (253)

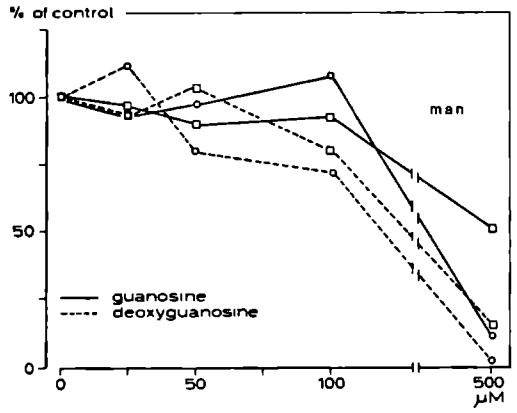


Fig. 8.3. Influence of deoxyguanosine and guanosine on thymidine (o) and uridine (□) incorporation of human PHA-stimulated lymphocytes. Values are means of 3-5 experiments. Variation coefficient of all values did not exceed 30%. Thymidine and uridine incorporation were measured at 51 hr, incorporation values are given in Fig. 8.2.

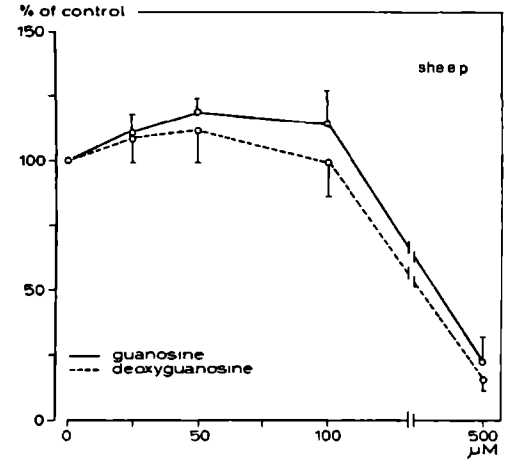


Fig. 8.4. Influence of deoxyguanosine and guanosine on thymidine incorporation of ovine PHA-stimulated lymphocytes. Values are means ± SD of 3-5 experiments. Thymidine incorporation was measured at 51 hr, incorporation values are given in Fig. 8.2.

and Snyder et al. (343) is comparable with our value. For crude and purified PNP preparations from other sources (erythrocytes, hepatoma cells, leukemic granulocytes and murine spleen) Km values for inosine ranging from 18 to 300 µM have been reported (30, 107, 199, 228, 317, 350, 412, 413). The Km value for deoxyguanosine in human T-lymphocytes (73) is comparable with our values in unseparated human and ovine lymphocytes. In human erythrocytes and leukemic granulocytes (350, 412) Km values for guanosine and deoxyguanosine ranging from 32-240 µM were found. With extracts from these cell types

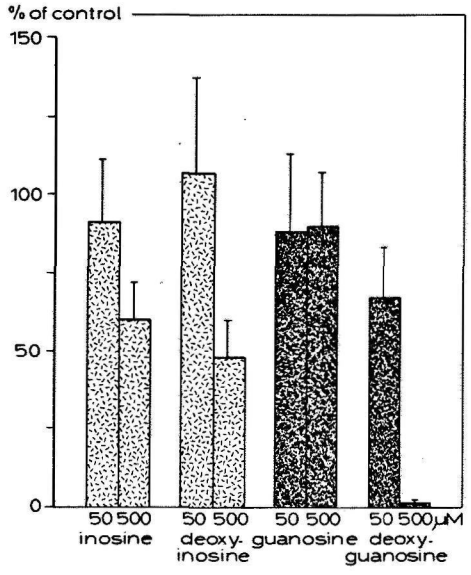


Fig. 8.5. Influence of inosine, deoxyinosine, guanosine and deoxyguanosine on thymidine incorporation of rat Con-A stimulated thymocytes. Values are means ± SD of 4 experiments. Thymidine incorporation was measured at 51 hr and ranged from 42 327-58 969 dpm for control Con-A stimulated cultures and from 1334-1729 dpm for cultures without Con-A.

the activity with deoxyguanosine is about half of the activity with inosine. We found the same with lysates of human lymphocytes (Table 8.1) and intact human lymphocytes and rat thymocytes (Table 8.3). North et al. (253) also found a higher activity of PNP with inosine as a substrate for extracts of human lymphocytes.

Activities of deoxyguanosine kinase in human lymphocytes reported by other investigators (46, 253, 254, 314) are comparable with our results. No data are available for the other species. Various Km values for deoxyguanosine have been reported for the purified calf thymus enzyme ranging from about 200 µM (181, 203) to 1.1 mM (205) and 3 mM (201). For human thymus and murine thymocytes Km values of 400 and 80 µM, respectively, were reported (46, 216), which are below our values in human lymphocytes and rat thymocytes. In calf thymus deoxycytidine was a better substrate for the enzyme than deoxyguanosine (203). In human thymus comparable activities and kinetics were found for deoxyguanosine and deoxyadenosine (46). With extracts from human lymphocytes the phosphorylation rate of deoxyguanosine was higher than that of deoxyadenosine, but the Km value for deoxyadenosine was much lower (chapter 5). All the purified enzymes did not catalyze guanosine phosphorylation, but lysates

of human and ovine lymphocytes contained a guanosine phosphorylating activity. The rate of phosphorylation of guanosine was below the lower limit of detectability with extracts of rat thymocytes. With extracts of human and ovine lymphocytes the guanine nucleotides will probably not be formed from guanosine by phosphorylysis to guanine and subsequent conversion by HGPRT, since the endogenous lymphocyte pool of PRPP delivers only about 10 pmol PRPP to the incubation mixture (chapter 2) and PRPP synthesis will not be likely because no phosphate and ribose 5-P are added to the enzyme assay. Furthermore guanine did not influence phosphorylation of guanosine and deoxyguanosine.

Studies on the metabolism of deoxyguanosine by intact cells have been limited to the measurement of dGTP that accumulates in human lymphocytes (67, 195, 362), human and murine thymocytes (49, 195), mouse lymphoma cells (53, 127, 389) and human T-lymphoblast lines (49, 235, 414). Only Hunting et al. (174) measured most metabolites in CHO cells that can be formed from deoxyguanosine. We confirmed the results of these authors that deoxyguanosine can be phosphorylated, and in addition is used for DNA synthesis. Furthermore we demonstrated that the rate of phosphorylysis exceeds the rate of phosphorylation, except in ovine lymphocytes at 17 µM deoxyguanosine. Chan (53) demonstrated with murine S-49 cells that deoxyguanosine does not markedly elevate the GTP level, but caused a sharp rise in the dGTP concentration. Hypoxanthine did not influence these changes.

In our cell systems hypoxanthine and guanine inhibited the phosphorylation of guanosine and deoxyguanosine. The residual activity in the presence of hypoxanthine will predominantly represent real deoxyguanosine and guanosine kinase activity, since hypoxanthine suppresses incorporation via guanine. A small amount of inosine may be phosphorylated directly since the non-radioactive hypoxanthine did not inhibit all incorporation of inosine into nucleic acids. Müller et al. (239) found a comparable utilization rate of hypoxanthine and guanine in intact human lymphocytes as we did for hypoxanthine. This route accounts mainly for the incorporation of inosine. The existence of an inosine kinase in mammalian tissues is still debatable (24, 42, 46, 216, 265).

The low rate of inosine incorporation correlates with the minor effects of inosine at concentrations up to 1 mM on PHA-stimulation of human lymphocytes that were also found by other investigators (106, 119, 120, 254, 279) and with the minor effects on cell growth

of several kinds of lymphoma cells (47, 53, 127, 254). With PHA-stimulated porcine lymphocytes 100 μM inosine or hypoxanthine even increased thymidine incorporation more than 3-fold (chapter 7). Deoxyinosine had an inhibitory effect on PHA-stimulation of human lymphocytes (48, 254, and Fig. 8.2). The growth of a HGPRT-deficient human lymphoblastoid cell line was unaffected by either inosine or guanosine, but inhibited by deoxyguanosine and deoxyinosine (46). No differences in the effects of guanosine and deoxyguanosine on thymidine, uridine and/or leucine incorporation were also found by Sakura et al. (314) and Cowan et al. (73). Deoxyguanosine was always more inhibitory than guanosine in human lymphocytes (73, 254, 314, and Fig. 8.3). Ochs et al. (254) and Cohen et al. (67) found comparable effects of guanosine and deoxyguanosine on thymidine incorporation of PHA-stimulated human lymphocytes as we. Sakura et al. (314) found only inhibition by guanosine in T-cells and not in B-cells and a higher sensitivity to deoxyguanosine in T-cells. The rate of inhibition by guanosine and deoxyguanosine reported by Cowan et al. (73) was lower, probably because they measured thymidine incorporation after 7 days. Inhibition by adenosine and deoxyadenosine of thymidine incorporation was more pronounced at shorter incubation times (152, 221, chapter 7).

In contrast to comparable experiments with ADA inhibitors (chapter 7), it did not appear possible to differentiate between the effects of guanosine and deoxyguanosine by inhibitors of PNP, as formycin B and allopurinol riboside. These enzyme inhibitors themselves cause considerable inhibition of thymidine incorporation (73, 249, 258, 314, 413). A combination of formycin B and deoxyguanosine did not give higher inhibition than deoxyguanosine alone (73).

Ovine lymphocytes and rat thymocytes have not been used previously to study the effects of guanosine and deoxyguanosine. Although ovine lymphocytes have a very low PNP activity and incorporate guanosine and deoxyguanosine at a comparable rate as human lymphocytes, they show a comparable sensitivity to guanosine, but a lower sensitivity to deoxyguanosine. Since ovine lymphocytes have lower levels of GTP than human, equine and porcine lymphocytes (chapter 9), they appear to differ in regulation of guanine nucleotide metabolism.

The sensitivity of rat thymocytes is lower than that found with human thymocytes (67), but comparable or higher than with PHA- or Con-A stimulated human lymphocytes (67, Fig. 8.3). The different sensitivity to guanosine and deoxyguanosine can be explained by the fact that rat thymocytes can only phosphorylate guanosine to a low

extent, but have a higher capacity to phosphorylate deoxyguanosine. Rat thymocytes appear to be a useful system to study the effects of deoxyguanosine, since precursors of the T-cells may be primarily affected in children with PNP deficiency (67). Furthermore the T and B lymphoblasts that are frequently used to study the effects of purine deoxynucleosides are rapidly growing cells. Spontaneously dividing human thymocytes are more sensitive to deoxyguanosine than PHA-stimulated thymocytes (67), but purine deoxynucleosides are also toxic to non-dividing peripheral lymphocytes and thymocytes (195). Therefore the effects of deoxynucleosides are not only mediated by inhibition of ribonucleotide reductase by accumulated dNTP, but one or more other mechanisms are involved. One of these mechanisms may be inhibition of methylation reactions caused by accumulation of SAH (144). This mechanism can play a role in PNP deficiency (146, 190) since inosine can inhibit SAH hydrolase in the presence of phosphate (146). Other possible mechanisms involve inhibition of PRPP synthesis by accumulated nucleotides (chapter 3) and inhibition of pyrimidine nucleotide synthesis (110, chapter 4). The use of rat thymocytes, with their low PNP activity and relatively high sensitivity to deoxyguanosine, may help to get further insight in the mechanism of purine deoxynucleoside toxicity.

8.5. Summary

1. Activity of PNP in lymphocytes of man was higher with inosine than with guanosine and deoxyguanosine as substrates. Ovine lymphocytes showed a comparable PNP activity with inosine, guanosine and deoxyguanosine. The activity is about 5% of that in human lymphocytes. Rat thymocytes have a comparable, low PNP activty. 2. Km values for inosine and guanosine in ovine lymphocytes (about 50 µM) were lower than in human lymphocytes (about 100 µM). Km values for deoxyguanosine were comparable (about 100 µM) in the cells of the three species. 3. The rate of phosphorylation of deoxyguanosine was much lower than the rate of phosphorylysis in extracts of human and ovine lymphocytes and rat thymocytes. This was also found with intact cells. 4. Deoxyguanosine, guanosine and inosine were incorporated by intact cells into nucleotides and nucleic acids. The incorporation of deoxyguanosine and guanosine was at least partially due to phosphorylysis and subsequent conversion by HGPRT. The incorporation of inosine appeared to be completely due to this route. 5. Inosine (0.5 mM) did not inhibit thymidine incorporation of PHA-

stimulated human and ovine lymphocytes. With both species the same concentration of deoxyinosine resulted in 50% inhibition. Guanosine and deoxyguanosine (0.5 mM) inhibited almost completely thymidine incorporation. Thymidine incorporation of Con-A stimulated rat thymocytes was hardly inhibited by 0.5 mM inosine, deoxyinosine and guanosine, but 50 µM and 0.5 mM deoxyguanosine caused 25% and complete inhibition, respectively.

Chapter 9

CONCENTRATION OF NUCLEOTIDES AND DEOXYNUCLEOTIDES IN PERIPHERAL AND PHA-STIMULATED MAMMALIAN LYMPHOCYTES

EFFECTS OF ADENOSINE AND DEOXYADENOSINE

9.1. Introduction

Measurement of intracellular pools of nucleotides is an important tool in the study of regulation of cellular metabolism. Pools of ribonucleotides and deoxyribonucleotides are disturbed in erythrocytes and lymphocytes of patients with ADA deficiency associated with SCID (85, 175, 318) and in lymphoblasts of leukemia patients (79). Cell systems that are incubated with adenosine or deoxyadenosine with or without ADA inhibitors show alterations in ribonucleotide and deoxyribonucleotide pools (47, 49, 173, 262).

Several differences in purine and pyrimidine metabolism of erythrocytes of various mammalian species exist (363, 368, chapter 5) and may be associated with marked differences in adenine and guanine nucleotide pools of these erythrocytes (40, 222). However, in erythrocytes no nucleotide pools for the synthesis of DNA and RNA have to be maintained.

Equine and porcine lymphocytes have a low ADA activity (chapter 5) comparable to that in lymphocytes of patients with ADA deficiency (261). Lymphocytes of horse and pig also show other marked differences in the enzymes of purine and pyrimidine metabolism (chapters 2, 3, 5) in comparison to that of man. Furthermore ovine lymphocytes and rat thymocytes have a very low PNP activity (chapter 5, 8). Therefore it would be of interest to measure the concentrations of ribonucleotides in the lymphocytes of man, horse, pig and sheep and in rat thymocytes with the same method. The use of advanced HPLC techniques (79) permitted measurement of all pyrimidine and purine nucleotides in one single run.

Adenosine and deoxyadenosine are toxic to many kinds of cells (138, 139), including human and equine lymphocytes (221, 223, chapter 7) but these nucleosides increased uridine, thymidine and leucine incorporation of PHA-stimulated porcine lymphocytes (chapter

7). Prelimary experiments indicated that dATP concentrations were increased with lymphocytes of man, horse and pig after incubation with deoxyadenosine and PHA. With a sensitive dNTP assay (172) we measured dATP and TTP pools in peripheral and PHA-stimulated lymphocytes with and without adenosine or deoxyadenosine. Furthermore the ATP concentration was measured in these cells by the luciferin-luciferase assay.

9.2. Materials and methods

9.2.1. Materials

The origin of chemicals used for HPLC is described previously (79). D(-)luciferin, luciferase (from Photinus pyralis), poly d(A-T), DNA polymerase I (from E. coli), TTP and dATP were from Boehringer, Mannhein, FRG. Deoxy{8-^{3}H}TTP and deoxy{8-^{3}H}ATP were from the Radiochemical Centre, Amersham, UK. Origin of the other chemicals and media needed for lymphocyte cultures is described in chapter 6. Blood samples were taken in heparinized bottles from adult horses (Equus caballus), pigs (Sus scrofa) and sheep (Ovis aries).

9.2.2. Preparation of extracts and HPLC analyses

Lymphocytes from the various species were isolated as described in chapter 5 and rat thymocytes as described in chapter 10. Nucleotides were extracted with ice-cold 0.4 M perchloric acid during 15 min. Proteins were precipitated by centrifugation and the supernatant was neutralized with an ice-cold mixture of 0.4 M KOH and 1 M K_2PO_4 (79). A Spectra-Physics Model SP 8000B HPLC system was used with a Partisil-10 SAX column. Nucleotides were separated using a multi-step gradient system (79).

9.2.3. dNTP assay

The dNTP assay described by Hunting & Henderson (172) was used with a slight modification. DNA pol I from E. coli (75 units/ml) was stored in 50 mM Tris-HCl(pH 7.8) with 12 mg BSA/ml; poly d(A-T) (2 units$_{A260}$/ml) in 100 mM Hepes (pH 7.4) with 0.1 mM EDTA. The reaction mixture (180 µl) contained 0.02 absorbance units (A260) poly d(A-T), 1.8 µmol $MgCl_2$, 1.8 µmol dAMP, 94 pmol {8-^{3}H}TTP (8 mCi/mmol) or deoxy{8-^{3}H}ATP (8 mCi/mmol), 0.66 mg BSA and 0-40 µl extract or

standard and was buffered with 100 mM Hepes (pH 7.4, 37°C). The reaction was started by addition of 0.75 unit DNA pol I and incubated in a shaking water-bath at 37°C. After 20 and 30 min 50 µl samples were taken and pipetted into a well of a 96-well microtitertek plate containing 100 µl of ice-cold TCA (7.5%) and 3% sodium pyrophosphate. The denatured high-molecular material was precipitated on a glass-fiber filter by the use of a cell harvester (Flow Laboratories, Irvine, UK) with 5 % ice-cold TCA during 15 sec. The filters were washed with distilled water, dried and radioactivity was measured as described in chapter 6.

9.2.4. Luciferin-luciferase assay

The concentration of ATP was also measured with a luciferin-luciferase assay in a Lumac cell-tester M 1030. D(-)luciferin (1 mM) was always freshly prepared in 0.1 M Tris-acetate (pH 7.5). A concentrated luciferase suspension (2.32 mg/ml in 0.5 M Tris-acetate, pH 7.5) could be stored at -20°C for several months without detectable loss of activity. The cuvet contained 100 µl 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and the extract (0-10 µl). Reaction was started by adding 50 nmol D(-)luciferin and 0.46 µg luciferase in 100 µl of a solution containing 50 mM Tris-HCl, 100 mM Tris-acetate (final pH 7.5) and 5 mM $MgCl_2$. The temperature of the reaction should be kept constant and not exceed 20°C. Luminiscence of the first 5 sec was integrated. ATP concentrations could be calculated from a calibration curve or by use of an internal standard of ATP that was added to the extract when it was measured for the second time.

9.2.5. Cell cultures

Cell cultures were performed as described in chapter 6. At 24 and 48 hr after starting the cultures the cells of several wells were pooled, centrifuged and extracted as described above. Thymidine incorporation was measured at 27 and 51 hr after starting the cultures as described in chapter 7. A concentration of 2.6 µM {6-^{3}H}-thymidine (3.3 Ci/mmol) was present during the last 6 hr of culture.

9.3. Results

9.3.1. Concentrations of ribonucleotides

Table 9.1. Concentrations of ribonucleotides in PBL of man, horse, pig and sheep and in rat thymocytes

Nucleotide	Man (7)	Horse (10)	Pig (10)	Sheep (6)	Rat (5)
AMP	40 ± 20	38 ± 26	43 ± 11	31 ± 11	70 ± 25
ADP	307 ± 70	154 ± 49	475 ± 201	116 ± 70	95 ± 14
ATP	904 ± 129	785 ± 229	1107 ± 210	461 ± 247	237 ± 33
GMP	10 ± 4	10 ± 5	13 ± 7	6 ± 4	14 ± 3
GDP	64 ± 28	36 ± 20	67 ± 31	32 ± 21	32 ± 7
GTP	180 ± 60	150 ± 58	205 ± 29	42 ± 26	80 ± 11
CDP	13 ± 8	15 ± 9	16 ± 9	9 ± 4	11 ± 4
CTP	38 ± 13	31 ± 11	34 ± 15	22 ± 12	27 ± 5
UDP	20 ± 10	15 ± 7	15 ± 5	11 ± 6	24 ± 6
UTP	142 ± 31	118 ± 39	96 ± 38	83 ± 26	53 ± 8
NAD^+	102 ± 89	66 ± 27	91 ± 50	73 ± 38	105 ± 19
UDP-glucose	25 ± 8	23 ± 7	26 ± 10	17 ± 6	-

Values (in pmol/10^6 cells) are means ± SD for the number of individuals indicated within parentheses.

Concentrations of all ribonucleotides in PBL and thymcoytes were determined with HPLC. Qualitative identification, quantitation and recovery of nucleotides were previously described (79). ATP determinations with luciferin-luciferase assay correlated well with the data of HPLC. The procedures for the isolation of lymphocytes and extraction were comparable for the various species (chapter 5), thus differences found can considered to be species-related. ATP was the most predominant ribonucleotide in lymphocytes of all species and in rat thymocytes (Table 9.1). Its concentration was highest in lymphocytes of pig and lowest in rat thymocytes. The concentration of ADP was also highest in porcine lymphocytes. The concentration of GTP was comparable in lymphocytes of man, horse and pig, but lower in those of sheep and in rat thymocytes. The concentrations of the other purine and of the pyrimidine nucleotides were comparable between the lymphocytes of the various species.

The TP/DP ratios and the energy charge give indication on the metabolic state of the cell. Equine lymphocytes showed the highest

Table 9.2. Energy charge and nucleoside triphosphate/nucleoside diphosphate ratios in mammalian lymphocytes and rat thymocytes

Species	Parameter	Ribonucleotides			
		Adenine	Guanine	Uracil	Cytosine
Man (7)	TP/DP	2.7 ± 0.7	3.2 ± 1.4	8.4 ± 2.9	5.8 ± 3.8
	EC	0.84 ± 0.03	0.84 ± 0.07	0.91 ± 0.09	0.88 ± 0.05
Horse (10)	TP/DP	5.4 ± 1.6	6.2 ± 2.7	10.5 ± 4.1	3.0 ± 1.1
	EC	0.88 ± 0.03	0.87 ± 0.07	0.94 ± 0.04	0.87 ± 0.04
Pig (10)	TP/DP	2.6 ± 0.7	3.7 ± 1.1	6.0 ± 3.5	3.2 ± 2.3
	EC	0.83 ± 0.03	0.83 ± 0.06	0.91 ± 0.04	0.87 ± 0.06
Sheep (6)	TP/DP	4.7 ± 2.2	4.4 ± 1.7	8.4 ± 4.2	2.6 ± 0.8
	EC	0.84 ± 0.10	0.86 ± 0.05	0.94 ± 0.02	0.85 ± 0.03
Rat (5)	TP/DP	2.5 ± 0.3	2.6 ± 0.3	2.3 ± 0.6	2.6 ± 0.9
	EC	0.71 ± 0.05	0.76 ± 0.02	0.86 ± 0.02	0.86 ± 0.04

Values are means ± SD and are calculated from nucleotide concentrations. The number of individuals is given within parentheses. EC, energy charge (TP + ½ DP / TP + DP + MP); TP/DP, nucleoside triphosphate/nucleoside diphosphate ratio.

ATP/ADP and GTP/GDP ratio (Table 9.2). The ratios for the other nucleotides were comparable in lymphocytes of the four species; the highest ratio was found for the uracil nucleotides. The energy charge for the purine and cytosine nucleotides was comparable in lymphocytes of the four species. The highest energy charge was found with uracil nucleotides.

9.3.2. Concentrations of dNTP

TTP and dATP were determined with the DNA polymerase assay. In order to check stability of dNTP under experimental conditions standard amounts of dATP and TTP were added to the cell suspension before extraction and to the reaction mixture. The recovery of the added dNTP was in both cases higher than 90%. The DNA polymerase reaction was proportional with the amount of extract. The increase of the amount of radioactivity incorporated to the poly d(A-T) template was comparable to that measured in the calibration curve after cor-

Table 3 Concentrations of dATP and TTP in peripheral and PHA-stimulated mammalian lymphocytes

Time (hr)	PHA		Man		Horse		Pig	
			dATP	TTP	dATP	TTP	dATP	TTP
0		Mean	1.7 ± 0.6 (7)	1.7 ± 1.1 (7)	0.7 ± 0.3 (7)	1.0 ± 0.8 (7)	2.0 ± 1.4 (9)	1.0 ± 0.5 (9)
		Range	0.9 - 2.3	0.9 - 4.0	0.4 - 1.2	0.1 - 2.3	0.8 - 4.3	0.4 - 1.8
24	-	Mean	2.4 ± 1.9 (5)	3.2 ± 1.8 (6)	0.8 ± 0.4 (6)	1.1 ± 1.1 (4)	0.9 ± 0.5 (7)	1.3 ± 0.6 (8)
		Range	1.9 - 6.2	1.5 - 6.0	0.3 - 1.5	0.1 - 2.6	0.3 - 1.8	0.2 - 2.1
	+	Mean	1.6 ± 1.2 (5)	2.8 ± 1.6 (6)	1.0 ± 0.6 (7)	1.1 ± 1.0 (6)	1.9 ± 1.4 (8)	1.5 ± 0.7 (8)
		Range	0.4 - 3.6	1.0 - 5.7	0.2 - 2.3	0.2 - 2.5	0.4 - 4.6	0.8 - 2.5
48	-	Mean	2.8 ± 2.1 (6)	3.0 ± 1.8 (6)	1.2 ± 1.0 (5)	- (5)	0.7 ± 0.3 (6)	0.8 ± 0.5 (6)
		Range	1.2 - 7.0	1.1 - 8.4	0.6 - 2.9	ND - 1.9	0.3 - 1.0	0.2 - 1.6
	+	Mean	3.9 ± 2.7 (6)	3.1 ± 2.3 (6)	1.1 ± 0.9 (7)	1.6 ± 0.8 (6)	1.2 ± 0.8 (8)	1.7 ± 0.6 (8)
		Range	1.3 - 8.7	0.5 - 6.6	0.2 - 2.5	0.4 - 2.9	0.4 - 2.9	0.9 - 2.4

Values (in pmol/10^6 cells) are means ± SD for the number of individuals indicated within parentheses. Most cultures were from the same lymphocyte preparation. ND, not detectable.

correction for the blancs. Values presented in Tables 9.3 and 9.5 were not corrected for loss during the assay and some dilution of the radioactive label by the endogenous dNTP. This dilution could be neglected because endogenous pools of TTP and dATP were always less than 5% of the added radioactive amount, even when dATP concentrations were markedly elevated.

Concentrations of dATP and TTP in peripheral and PHA-stimulated lymphocytes are given in Table 9.3. Only cultures were used that showed an appropriate thymidine incorporation, when measured after 2 days. The stimulation index was higher than 37 for human, higher than 10 for equine and higher than 16 for porcine lymphocytes. Thymidine incorporation amounted to more than 30 000 dpm for human, to more than 12 000 dpm for equine and to more than 70 000 dpm for porcine PHA-stimulated lymphocytes. No differences in dATP and TTP concentrations were noted between PBL and cultured lymphocytes of horse and pig with and without PHA. In cultured human lymphocytes the concentration of TTP seemed to be higher than in PBL. The concentration of dATP in rat thymocytes was 1.6 ± 0.4 and that of TTP 4.6 ± 0.7 pmol/10^6 cells (means ± SD of 5 rats)

9.3.3. Concentration of ATP in cultured cells

The ATP concentration was measured in PBL and cultured cells by the luciferin-luciferase assay. This assay is much faster and more sensitive than the HPLC method for single ATP determinations. With all species the ATP concentrations decreased during culturing as well in the presence as in the absence of PHA (Table 9.4). This decrease was higher with human and equine lymphocytes than with porcine lymphocytes. Cultured porcine lymphocytes contained the highest ATP concentration, as well relatively as absolutely.

Table 9.4. Influence of PHA-stimulation on ATP concentration

Time	PHA	Man (5)	Horse (4)	Pig (6)
24 hr	-	50 ± 9	44 ± 7	74 ± 28
	+	57 ± 13	52 ± 11	75 ± 30
48	-	37 ± 11	30 ± 2	49 ± 16
	+	50 ± 13	39 ± 12	53 ± 15

Values (in % of the concentration at the start of the culture) are means ± SD for the number of cultures given within parentheses.

Table 9.5. Influence of adenosine and deoxyadenosine with or without EHNA on the concentrations of ATP, dATP and TTP in PHA-stimulated mammalian lymphocytes

Nucleotide	Species	Time (hr)	Relative concentrations of nucleotides in the presence of							
			Adenosine				Deoxyadenosine			
			100 µM	50 µM	25 µM	25 µM + EHNA	100 µM	50 µM	25 µM	25 µM + EHNA
ATP	Man	24	109	113	101	120	115	140	122	92
		48	109	107	128	117	141	137	120	47
	Horse	24	158	143	160	184	159	164	142	135
		48	62	104	109	98	114	128	101	82
	Pig	24	158	171	182	202	183	181	145	178
		48	147	151	147	158	161	168	167	121
dATP	Man	24	224	120	118	101	186	153	152	325
		48	67	118	93	90	243	80	65	266
	Horse	24	80	92	89	106	237	236	112	1203
		48	145	79	107	60	73	70	54	801
	Pig	24	76	108	158	106	547	211	138	936
		48	112	104	100	69	454	223	255	966
TTP	Man	24	128	103	134	130	121	112	91	109
		48	120	96	75	81	114	99	123	96
	Horse	24	107	153	142	87	100	113	129	116
		48	90	112	140	105	92	158	105	101
	Pig	24	97	101	99	93	74	81	104	81
		48	94	89	97	102	107	119	83	90

Values (means of 4-6 separate experiments) are given in % of the concentration of the nucleotide in PHA-stimulated lymphocytes in the absence of metabolites at the indicated hr. These concentrations are given in Table 9.3. SD was always lower than 40% of the mean. The concentration of EHNA was 10 µM.

9.3.4. Influence of adenosine and deoxyadenosine

In chapter 7 we described the influence of adenosine and deoxyadenosine with and without EHNA on PHA-stimulated thymidine, uridine and leucine incorporation. In some of these and similar cultures we measured the concentrations of ATP, TTP and dATP (Table 9.5). Adenosine or deoxyadenosine did not alter in most cases the ATP concentration in PHA-stimulated cells cultured for 24 and 48 hr. In equine and porcine lymphocytes the concentration of ATP was relatively higher in the presence of adenosine. With human lymphocytes the ATP concentration was lower in the cultures with deoxyadenosine and EHNA. Addition of adenosine in various concentrations did not affect dATP concentration. Deoxyadenosine at 25 and 50 µM did not affect dATP concentration with human lymphocytes and increased it in porcine lymphocytes; at 100 µM deoxyadenosine, dATP concentration was markedly increased in lymphocytes of all species at 24 hr and in human and porcine lymphocytes also at 48 hr. In the presence of deoxyadenosine and EHNA an increase in dATP was found with all species that was higher with porcine and equine lymphocytes. TTP concentration was not significantly affected by adenosine or deoxyadenosine.

9.4. Discussion

Studies on concentrations of nucleotides in human lymphocytes were mostly limited to ATP and ADP. Reports on all purine and pyrimidine nucleotides are rare. The concentrations of ATP reported by Schmalstieg et al. (318), Wolberg et al. (416) and De Abreu et al. (79) are slightly lower than our value, that is comparable to that of Nelson et al. (246) and Skupp et al. (337). Chapman et al. (57) reported very high concentrations of all three adenine nucleotides. Nelson et al. (246) and Skupp et al. (337) reported comparable values for the ADP concentration as we, but Schmalstieg et al. (318) and De Abreu et al.(79) reported a lower ADP concentration. Values for the concentrations of GTP, GDP, CTP and UTP by other investigators (79, 246, 318, 337, 416) are comparable to our values. Ratios of ATP/ADP previously reported (246, 318, 337) are comparable; the energy charge is not given, but appears to be in the same range. De Abreu at al. (79) gives slightly higher values. The concentration of NAD^+ reported by Chapman et al. (57) and Blomquist et al. (28) is comparable to our value.

The concentration of ATP in lymphocytes of adult horses is comparable to our value (223). No data are available on lymphocytes of

the other animals.

Although large differences exist in purine and pyrimidine metabolism of the species investigated (chapters 2,5,8), no such differences are found in the concentrations of the nucleotides. Equine lymphocytes have a very low capacity of pyrimidine de novo synthesis in their lymphocytes, but the capacity of the salvage pathway seems to be high enough (370) to maintain pyrimidine nucleotides in the same concentration as with the other species. Only with the purine nucleotides some significant differences were found, but they can not be explained by the differences found in purine metabolism (chapter 5). The differences in the nucleotide contents of the lymphocytes of the various species are smaller than the differences found for their erythrocytes (40). Furthermore in lymphocytes of all species the concentrations of the triphosphates are always higher than those of the diphosphates in contrast to in erythrocytes of some species (40).

Most authors found a considerable variation in the concentrations of dNTP in lymphocytes of individuals. These variations are probably due to the fact that the lymphocyte populations differ in their extent of activation. The concentration of dATP has been measured more frequently than that of the other dNTP. Our values for the dATP and TTP concentration in human lymphocytes are in the same range as those found by other investigators (85, 175, 195, 261).

Munch-Petersen et al. (242) and Tattersall et al. (261) reported an increase in the concentration of dATP after culturing of human lymphocytes in the presence of PHA. However, from their and our data (Table 9.3) this conclusion does not seem to be justified. The absolute concentrations of TTP and dATP in PHA-stimulated lymphocytes are in the same large range. The increase in the concentration of TTP seemed to be significant. This increase is parallelled by a higher thymidine and uridine incorporation (242, chapter 7) and a higher activity of enzymes involved in the synthesis of precursors of DNA as thymidine kinase, deoxycytidine kinase and deoxycytidylate deaminase (165, 243, 266) and of DNA polymerase (384). With unstimulated and stimulated lymphocytes of horse and pig we found no significant increase in the concentrations of TTP and dATP.

The decrease in ATP concentration during culturing is probably the result of nutrient exhaustion of the culture medium, since the ATP concentration decreases as well with control cells as with PHA-stimulated cells. This was also found with equine lymphocytes (223) and with cultured mammalian cell lines (292, 406).

Incubation of lymphocytes with adenosine helped to maintain higher ATP levels with lymphocytes of horse and pig (Tables 9.4 and 9.5), but with human lymphocytes only the combination adenosine plus coformycin increased the ATP concentration (132), in contrast to adenosine plus EHNA (Table 9.4). A higher ATP concentration after incubation with adenosine was also found for other cell types (127, 184, 292).

Adenosine did not significantly influence the concentration of dNTP in human, equine and porcine lymphocytes (132, Table 9.5). The concentration of dATP in human lymphocytes was increased and the concentrations of dGTP, dCTP and TTP were drastically reduced at 48 hr in the presence of adenosine and coformycin (132). Adenosine decreases the concentration of PRPP in lymphocytes and lymphoblasts (343, 344, chapter 7).

Accumulation of dATP after incubation of cells with deoxyadenosine in the presence or absence of an ADA inhibitor is a common feature for lymphocytes (29, 195, 262), human, murine and equine thymocytes (49, 195, 223), lymphoblast cell lines (47, 49, 145, 235,388 391, 414), hepatoma cells (130), human fibroblasts (111), Hela cells (210) and CHO cells (173). The accumulation of dATP is higher with T-cell lines than with B-cell lines (49, 235). Furthermore dATP is elevated in erythrocytes and lymphocytes of patients with ADA deficiency associated with SCID (69, 85, 175, 254). In most cases this accumulation is accompanied by inhibition of cell growth. Our results with porcine lymphocytes indicate that accumulation of dATP is not always associated with inhibition of cell proliferation. The high concentration of dATP, that is present in porcine lymphocytes after incubation with deoxyadenosine, is associated with an increased thymidine, uridine and leucine incorporation (chapter 7), and the concentration of TTP is not affected. However, no increase in dATP or TTP levels is observed after incubation of porcine lymphocytes with adenosine, although all incorporation values also increased (chapter 7). Probably the normal pools of dATP and TTP are high enough under this condition for the increased DNA synthesis and these dNTP are delivered at a higher rate via ribonucleotide reductase. The dATP formed from deoxyadenosine can be part of a pool that is not used for DNA synthesis. With human lymphocytes is has been demonstrated that TTP formed via the salvage pathway (thymidine kinase) forms a separate pool (358). The existence of such a pool for dATP could explain why with porcine lymphocytes accumulation of dATP is not associated with inhibition of cell growth. Another explanation may be

that the inhibitory concentration of dATP for ribonucleotide reductase is different for the various species. A 2-5 fold increase of dATP concentration by 50-100 µM deoxyadenosine is associated with a 5-fold increased thymidine incorporation in porcine lymphocytes, while a comparable concentration of dATP caused by 25 µM deoxyadenosine and 10 µM EHNA is associated with a partially to completely inhibited incorporation in equine and human lymphocytes, respectively (chapter 7). An increase of the dATP concentration does not lead in all cell types to an inhibition of the GDP and CDP reduction catalyzed by ribonucleotide reductase (173, 390). Furthermore the effects on mitogen-induced proliferation of human lymphocytes (387) and rat thymocytes (379) are most pronounced when added at the first day of culture, when DNA synthesis has not yet been increased. Even unstimulated lymphoid cells accumulate dATP and are killed by the presence of deoxyadenosine (195). Therefore some other mechanism(s) besides inhibition of ribonucleotide reductase are involved in the disturbance of cell growth and function by deoxyadenosine. Purine and pyrimidine de novo synthesis are not affected significantly by a combination of deoxyadenosine and coformycin (374). The effects of deoxyadenosine on PRPP synthesis have not yet been studied, but dAMP and dATP can inhibit PRPP synthetase (chapter 3). The effects of deoxyadenosine could also be mediated by inhibition of methylation reactions (144) or interference with energy metabolism.

9.5. Summary

1. Concentrations of purine and pyrimidine ribonucleotides were measured with HPLC in lymphocytes of man, horse, pig and sheep and in rat thymocytes. The concentration of ATP was highest in lymphoid cells of all species and about 850 pmol/10^6 cells in human and equine lymphocytes, higher in porcine and lower in ovine lymphocytes and rat thymocytes. The concentration of GTP was comparable in human, equine and porcine lymphocytes, but lower in ovine lymphocytes. The concentration of UTP and CTP were comparable in lymphocytes of the four species. 2. The energy charge for the purine and cytosine ribonucleotides did not show significant species differences and varied between 0.83 and 0.88 with the lymphocytes; the energy charge of the uracil ribonucleotides was higher. 3. ATP concentration was also measured in cultured lymphocytes of man, horse and pig with a luciferin-luciferase assay. During culturing with or without PHA the ATP concentration decreased in lymphocytes of all species.

4. The concentrations of TTP and dATP were measured with a DNA polymerase assay. In PBL the concentration of both dNTP varied between 0.1-4.3 pmol/10^6 cells . In cultured lymphocytes of man, horse and pig with or without PHA the concentrations of dATP and TTP were comparable to those in PBL. 5. ATP, TTP and dATP concentrations were also measured in PHA-stimulated lymphocytes of man, horse and pig after 24 and 48 hr culturing with and without adenosine or deoxyadenosine. ATP concentrations were relatively higher in porcine and equine lymphocytes in the presence of adenosine. TTP concentrations were not significantly altered in the presence of adenosine or deoxyadenosine. Addition of adenosine did not result in significant changes of the dATP concentration. Deoxyadenosine in the presence of EHNA increased the dATP concentration with all species 3-12 fold and caused in all cultures a decreased thymidine and uridine incorporation (chapter 7). However, with porcine lymphocytes deoxyadenosine without EHNA increased the dATP concentration 2-5 fold , but stimulated the thymidine and uridine incorporation about 5-fold (chapter 7). The latter results indicate that accumulation of dATP is not always associated with inhibition of cell proliferation.

Chapter 10

AGE-DEPENDENCY OF ADA AND PNP ACTIVITIES IN RAT SPLEEN AND THYMUS*

10.1. Introduction

Inherited deficiencies of the purine degrading enzymes, ADA and PNP, in human lymphocytes have been associated with SCID (122) and with severe T-cell dysfunction (123), respectively. ADA deficiency is not only associated with severe T and B cell dysfunction but also with an absent or a very small thymus (161). Absence or removal of the thymus at birth causes severe depletion in the lymphocyte population and serious immunological defects in the adult (304). Although ADA or PNP is deficient in all cells of affected individuals, only lymphoid organs show disturbances in development and function. Jackson et al. (182) reported marked differences in activities of the adenosine metabolizing enzymes ADA and AK between various rat tissues and found the highest ADA activity in the lymphoid organs. Barton et al. (15, 16) reported marked differences in activities of ADA and PNP between various stages of fetal T-cell differentiation and between various types of rat lymphoid cells. They postulate that ADA and PNP play an important role in lymphocyte differentiation by degradation of the potentially toxic nucleosides adenosine and deoxyadenosine. Jackson et al. (182) relate the high ADA activity in rapidly proliferating cells and organs like thymus to an increased protection against adenosine toxicity.

Since the development of immune function enters a critical phase at birth, we measured the activities of ADA, PNP and AK in thymocytes and splenocytes of rats of 0-423 days and compared kinetics of adenosine and deoxyadenosine deamination in 3- and 40-days old rats.

10.2. Materials and methods

10.2.1. Materials

Source of chemicals and composition of solutions are described in chapter 5. In all age groups both male and female Wistar rats were used.

*adapted from Peters et al. (273)

10.2.2. Preparation of thymocytes and splenocytes

Rats were killed by cervical dislocation. Thymus and spleen were immediately removed and placed in glass vials containing MEMS medium (Flow Laboratories, Irivine, UK) with Hepes buffer (pH 7). After removal of fat and connective tissue, organs were weighed and minced with a pair of scissors. All spleens and thymuses of older animals (60-423 days) were gently homogenized with a teflon Potter-Elvejhem homogenizer at 4°C (the intervening space was 0.5 mm). The homogenate was filtered over double cheese filter (pore size 25 µm). Thymuses of young animals could be dispersed on the filter. The filtrates were layered on a discontinuous Ficoll-Isopaque gradient (3 ml with a density of 1.077 and 1 ml with a density of 1.050) and centrifuged for 30 min at 400 g at 18°C. The cells at the interfase between the densities 1.077 and 1.050 were aspirated and washed twice with MEMS-Hepes. The cells (thymocytes and splenocytes) were counted in a hemocytometer. Some cytocentrifuge smears were prepared and stained for the detection of polymorphonuclear cells and monocytes. All cell preparations contained less than 4% of polymorphonuclear cells and monocytes. Viability was higher than 90% as determined with trypan blue exclusion.

10.2.3. Enzyme assays

All assays were performed on thymocytes and splenocytes from individual animals. For the studies of postnatal development (0-20 days) 3-5 litters of at least 6 animals were used. For cell extracts cells were suspended in 50 mM Tris-HCl (containing 1 mM EDTA, pH 7.4) to the appropriate cell concentration and lysed by sonication (8 bursts of 5 sec at 0°C with a Branson sonifier at maximal output). Activities of ADA, PNP and AK were determined as described in chapter 5. The reaction mixture for ADA contained an amount of thymocyte or splenocyte lysate equivalent to $2.5 - 30 \times 10^3$ cells and $25 - 190 \times 10^3$ cells, respectively. For the PNP assay these amounts were $6 - 18 \times 10^4$ cells. For the AK assay volumes of 7000 g supernatant equivalent to $1.5 - 3 \times 10^6$ cells and $0.25 - 1.0 \times 10^6$ cells, respectively were used. Protein was measured according to (212).

10.3. Results

With animals of all ages equal amounts of extracts were used

for each assay. All enzyme assays were linear with time, substrate concentration and amount of extract.

ADA showed an age-dependency with thymocytes and splenocytes (Fig. 10.1). The activity of ADA is highest in thymocytes of newborn rats and decreases at postnatal development. With animals older than 6 days no further change was found. With splenocytes ADA activity is low in newborn rats and gradually increases up to the age of 20 days. With maturation and ageing no further significant changes were found. At all ages ADA activity is higher in thymocytes than in splenocytes. During whole life the activity of ADA is higher than that of PNP in thymocytes, while in splenocytes the PNP actity is higher (Fig. 10.2). With thymocytes PNP does not show an age-dependency, while in splenocytes the activity of 40- and 60-days old animals is lower than in younger animals. Activity of AK in thymocytes was measured at 13, 20, 40 and 60 days old rats. No age-dependency was found, the mean ± SD for these 16 animals was 0.83 ± 0.36 nmol/hr per 10^6 cells. Activity of AK in splenocytes of 40-days old animals was 5.9 ± 1.1 nmol/hr per 10^6 cells (mean ± SD of 3 rats). When the results are expressed per mg protein, the activities ADA, PNP and AK show the same pattern as when they are expressed on base of 10^6 cells. The activities of ADA, PNP and AK expressed per mg protein in 40-days old animals are given in Table 10.1.

Activity of ADA measured in thymocytes of the same animal was higher with deoxyadenosine than with adenosine as a substrate. The ratios of the activities were 1.34 ± 0.10 and 1.21 ± 0.13 for 3- and 40-days old animals, respectively (mean ± SD for 4 and 7 animals, *respectively*). *The Km values of adenosine and deoxyadenosine for ADA* (Table 10.2) showed no significant differences in thymocytes between 3- and 40-days old rats and between adenosine and deoxyadenosine.

Table 10.1. Activities of ADA, PNP and AK in thymocytes and splenocytes of 40-days old rats

	Thymocytes	Splenocytes
ADA	23760 ± 3880	3265 ± 468
PNP	1494 ± 253	4799 ± 873
AK	462 ± 165	424 ± 25

Activities (in nmol/hr per mg protein) are means ± SD for 3-4 animals. Activity of AK was measured in 7000 g supernatants.

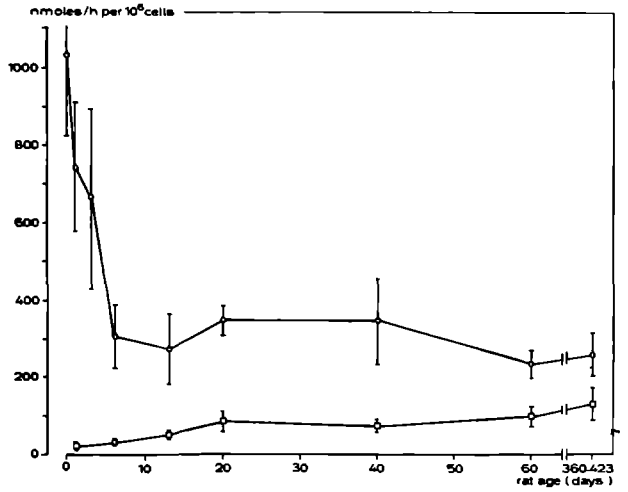


Fig. 10.1. Activity of ADA in splenocytes (□) and thymocytes (o) of rats of different ages. Activities are means ± SD for 8-14 rats of 0 to 40 days and 4-5 rats for the other age groups.

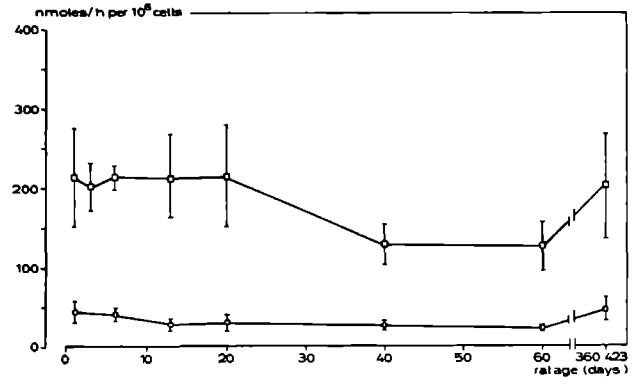


Fig. 10.2. Activity of PNP in splenocytes (□) and thymocytes (o) of rats of different ages. Activities are means ± SD for 8-15 rats of 1 to 20 days and 5-8 for the other age groups.

Table 10.2. Km values of adenosine and deoxyadenosine for ADA in thymocytes of 3- and 40-days old rats

Rat age	Adenosine	Deoxyadenosine
3 days	31.2 ± 10.5 (4)	28.3 ± 5.4 (4)
40 days	53.2 ± 19.9 (5)	36.8 ± 4.6 (5)

Values (in μM) are means ± SD for the number of animals indicated within parentheses.

10.4. Discussion

Activities of ADA and PNP found in splenocytes and thymocytes of 40-days old rats agree well with those measured by Barton et al. (15, 16). Activity of ADA in thymocytes reported by Cartier (52) is somewhat higher. Jackson et al. (182) reported comparable values for ADA and AK in spleen and thymus as we did.

Barton et al. (15, 16) postulate a reciprocal relationship between ADA and PNP activities in lymphoid cell types and at various stages of T-cell differentiation. However, activity of ADA in spleen increases in the postnatal period while activity of PNP does not increase. Similarly with thymocytes no increase in PNP activity is found when ADA decreases. Scholar et al. (322) reported a decrease of PNP in spleens of mice during ageing while ADA did not change.

The Km values of adenosine and deoxyadenosine for ADA in thymocytes are comparable with the values found in lymphocytes of various mammalian species (chapter 5). Deoxyadenosine will be deaminated most efficiently since activity of ADA is higher with deoxyadenosine and the Km of deoxyadenosine for deamination is much lower than for phosphorylation (109, chapter 5). The high ADA/AK ratio will ensure degradation of adenosine. In murine thymocytes adenosine and deoxyadenosine are deaminated predominantly at concentrations ranging from 0.1-1000 μM (345).

The ADA enzyme in thymus probably is the low MW type (MW 35 kD), since it has comparable kinetic properties as described by Hirschhorn & Ratech (159) for this enzyme; low Km values for both substrates and a higher deamination rate for deoxyadenosine. Furthermore with thymocytes and splenocytes EHNA, present in the AK assay, almost

completely inhibited deamination of adenosine. This gives evidence that the ADA isozyme in splenocytes probably also is of the low MW type, since a high sensitivity to EHNA is a property of this type (294, 296). Van der Weyden & Kelley (399) found that in human spleen ADA is predominantly of the low MW type and that no conversion activity is present.

Barton et al. (15, 16) found a prenatal increase of ADA activity in fetal thymocytes, but no difference between newborn and young adult rats, in contrast to our results (Fig. 10.1). The decrease in ADA activity in the thymus proceeds simultaneously with the appearance of TdT activity in cortical thymus cells in the first week after birth (31). Since ADA activity is predominantly located in cortical thymocytes (15, 16) a comparable change in cell population could be responsible for the decrease in ADA activity as for the appearance of TdT (31). The high activity of ADA in thymuses of newborn rats can result in a better protection to adenosine and deoxyadenosine toxicity as was observed in hepatoma cell lines with a high ADA activity (182). Therefore, the changes in ADA activity during postnatal development may have more important consequences for lymphocyte development than the changes observed in the activities of TdT (31) and ecto-5'-nucleotidase (37).

10.5. Summary

1. Activities of ADA and PNP were determined in thymocytes and splenocytes of rats of 0-423 days old. 2. Activity of ADA per cell is highest in thymocytes of newborn rats and decreases with postnatal development, while in splenocytes ADA activity increases. No significant age-dependency is found with PNP in thymocytes and splenocytes. 3. During whole life ADA activity is higher in thymocytes and PNP activity is higher in splenocytes. 4. With thymocytes ADA activity was higher with deoxyadenosine than with adenosine. The Km values of both nucleosides were comparable in 3- and 40-days old rats (about 30-40 μM).

Chapter 11

METABOLISM OF PURINE NUCLEOSIDES AND PRPP IN THYMOCYTES AND SPLENOCYTES OF VARIOUS MAMMALIAN SPECIES*

11.1. Introduction

Purine nucleosides interact with the immune system in man. First indications for this interaction were obtained by Giblett et al. (122, 123) who described the association of absence of two purine nucleoside-degrading enzymes with immune dysfunction. In man ADA deficiency was associated with SCID and deficiency of PNP with a severe T-cell dysfunction. The ADA deficiency was found in all tissues of affected children (153) but the lymphoid tissues showed relatively the lowest levels. The thymus not only showed very low ADA activities, but was very small or even absent in children with ADA deficiency. The age-dependency of ADA in rat thymocytes and splenocytes (15, 16, chapter 10) gives further evidence that ADA plays an important role in the development of immune function.

In chapter 5 we reported that lymphocytes of horse and pig have an ADA activity comparable to that in children with ADA deficiency and that ovine lymphocytes have a very low PNP activity. We used lymphocytes from these species to study the effect of the substrates of ADA and PNP on lymphocyte function (chapter 7-9). In order to get more insight into the role of ADA and PNP in development of immunologically potent cells we now measured the activities of these nucleoside catabolizing enzymes and those of adenosine and deoxyguanosine kinase in thymocytes and splenocytes of man, horse, pig, sheep, rat and mouse.

PRPP plays an important role in purine salvage pathways that use hypoxanthine and guanine, the products of PNP, as substrates. Previously we provided evidence that disturbances of PRPP metabolism could play a role in the metabolic effects of ADA and PNP deficiency (chapters 3 and 7). Therefore in this study we also examined the activity of PRPP synthetase and the concentration of PRPP in the various lymphoid cells.

*adapted from Peters et al. (276)

11.2. Materials and methods

11.2.1. Materials

Sources of chemicals and composition of solutions are described in chapters 5 and 10. Thymuses and spleens of horses (Equus caballus), pigs (Sus scrofa) and Texel sheep (Ovis aries) were obtained at local slaughterhouses from healthy animals. Wistar rats (Rattus norvegicus) and Swiss mice (Mus musculus) were obtained from the Central Animal Laboratory of the University. Human organs were obtained from children that died within two days after delivery after a pregnancy period of 30-40 weeks. Death causes did not concern the immune system. Thymocytes and splenocytes were prepared as soon as possible after death. With rats and mice this could happen immediately, with pigs and sheep within about 1-2 hr and with horses and children within 2-8 hr. One sample of human thymus was obtained at cardiac surgery of a man of 40 years.

11.2.2. Preparation of thymocytes and splenocytes

Preparation of thymocytes and splenocytes of rats is described in chapter 10. The MEMS-medium was replaced by 20 mM Hepes-buffered saline (pH 7.0), since cells that are isolated in MEMS-medium show PRPP synthesis during isolation because of the presence of phosphate (chapter 2). Organs of mice were processed in the same way. After removal of fat and connective tissue the organs of man, horse, pig and sheep were weighed, minced in small pieces and homogenized with a teflon Potter-Elvejhem homogenizer at $4^{o}C$ (the intervening space was 0.5 mm). The homogenate was filtered over a nylon sieve and the filtrates were layered on a Ficoll-Isopaque gradient with a density of 1.077 and centrifuged for 30 min at $18^{o}C$ at the same speed as used for lymphocytes of these species (chapter 5). The cells at the interfase (thymocytes and splenocytes) were washed twice with Hepes-buffered saline and counted with a hemocytometer.

11.2.3. Enzyme assays

All enzyme assays were performed on thymocytes and splenocytes from individuals. For preparation of lysates cells were suspended in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, to the appropriate cell concentration and lysed by sonication (8 bursts of 5 sec with

a Branson sonifier at maximal output). Only for the assay of PRPP synthetase cell lysates were prepared in 25 mM phosphate buffer (containing 1 mM EDTA, pH 7.4). Activities of ADA, PNP and AK were determined as described in chapter 5. Assay of deoxyguanosine kinase is described in chapter 8 and determination of activity of PRPP synthetase and concentration of PRPP in chapter 2. Washing with Hepes-buffered saline does not affect the assays of PRPP concentration and PRPP synthetase activity in comparison to the earlier used washings with Tris-buffered saline and PBS, respectively (chapter 2). The volumes of thymocyte and splenocyte lysate used are comparable to that used for lymphocytes. For the assays of AK and deoxyguanosine kinase a 7000 g supernatant was used. Protein was measured according to (212).

11.3. Results

11.3.1. Metabolism of purine nucleosides

All enzyme assays were proportional with time, substrate and amount of tissue material. Activities of ADA and PNP in thymocytes and splenocytes of the animals and the newborn babies are presented in Figs. 11.1 and 11.2. For comparison we also show the activities in PBL of adult mice and the previously reported activities in PBL of the other mammals (chapter 5). Since the organs of the babies were obtained after different gestation periods, and during maturation considerable changes in the activities of some enzymes occur in the rat (15, 16, chapter 10), some separate values are shown for man and horse in Figs. 11.1 and 11.2. The values were mediated when they were close to each other as e.g. the PNP activity of the babies. With man, horse and rat activities of ADA were comparable in PBL and splenocytes, with pig and sheep activity of ADA was higher in splenocytes. With all species, except the horse, activity of ADA was higher in thymocytes than in PBL. With pig and sheep activity of ADA in thymocytes was lower than in splenocytes. With all species activity of PNP is higher in splenocytes than in thymocytes. Activity of PNP was highest in human PBL and lowest in ovine thymocytes.

Activity of AK is comparable in thymocytes of all species (Table 11.1). With man, horse and pig activity of AK in thymocytes is comparable to that in splenocytes and PBL (chapter 5). Rat PBL have a higher activity of AK (9.2 ± 3.0 nmol/hr per 10^6 cells, mean ± SD of 4 individuals). The activity of deoxyguanosine kinase is highest in human thymocytes. In ovine splenocytes activity of deoxyguanosine

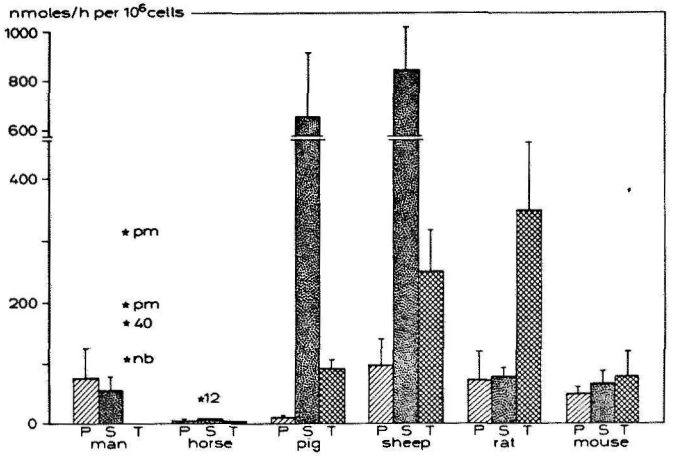


Fig. 11.1. Activity of ADA in PBL (P), thymocytes (T) and splenocytes (S) of various mammalian species. Enzyme activities (in nmol/hr per 10^6 cells) are means ± SD for the number of individuals as indicated in Table 11.3. 40, activity in thymus from a 40 year old man; pm, from a premature child; nb, from a newborn child; 12, in a spleen from a 12 year old horse.

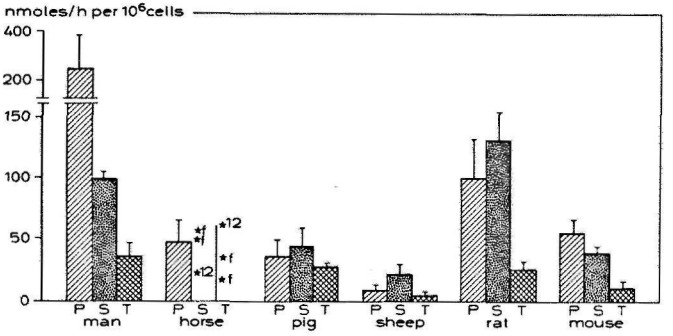


Fig. 11.2. Activity of PNP in PBL (P), thymocytes (T) and splenocytes (S) of various mammalian species. Enzyme activities (in nmol/hr per 10^6 cells) are means ± SD for the same number of individuals as indicated in Table 11.3. 12, activity in a spleen from a 12 year old horse; f, from a foal of 10 months old.

Table 11.1. Activities of AK and deoxyguanosine kinase (dGK) in thymocytes and splenocytes of various mammalian species

Species	Age	Thymocytes		Splenocytes
		AK	dGK	AK
Man	p.m.	0.58; 0.88		0.96 ± 0.44 (3)
	n.b.	0.67	1.26	2.52
Horse	10 mo	0.4; 1.1	N.D.	0.62; 0.99
	12 yr	1.5		3.45
Pig	6 mo	0.34 ± 0.03 (3)	N.D.	1.0 ± 0.4 (5)
Sheep	1 yr	1.3 ± 0.4 (3)	0.48 ± 0.08 (3)	2.4 ± 0.6 (4)
Rat	40 d	0.61 ± 0.11 (4)	0.10 ± 0.05 (8)	5.9 ± 1.1 (3)
Mouse	40 d	0.81 ± 0.36 (3)	N.D.	N.D.

Activities (in nmol/hr per 10^6 cells) are means ± SD for the number of individuals indicated within parentheses. Enzyme activities were measured in 7000 g supernatants. N.D., not determined, p.m., premature; n.b., newborn.

kinase was 0.34 ± 0.04 nmol/hr per 10^5 cells (mean ± SD of 3 determinations).

In order to get more insight into adenosine metabolism the ratio of ADA and AK activities (per 10^6 cells) was calculated in the experiments where ADA and AK were measured simultaneously. With all species studied the highest ADA/AK ratio was found in thymocytes, except with pig (Table 11,2). The ratio of ADA/AK was comparable between splenocytes and PBL of the same species except with horse and pig.

Since enzyme activities in many related studies were expressed per mg protein, we also based the activities of ADA, PNP and AK on this parameter (Table 11.3). The difference in ADA between PBL and thymocytes became more pronounced due to the low content of protein in thymocytes probably because these cells are relatively small. Since on the other hand splenocytes of pig and sheep contain a relatively high concentration of protein, the differences in activities between thymocytes and splenocytes of these animals are less pronounced.

Table 11.2. Ratios of the activities of ADA and AK in thymocytes, splenocytes and PBL of various mammalian species

Species	ADA/AK ratio in		
	Thymocytes	Splenocytes	PBL
Man	322 ± 41	64 ± 37	61 ± 31
Horse	5 ± 2	12 ± 4	2 ± 1
Pig	267 ± 32	623 ± 141	19 ± 14
Sheep	183 ± 40	103 ± 54	97 ± 57
Rat	600 ± 253	13 ± 4	11 ± 1
Mouse	94 ± 19	N.D.	N.D.

Values (means ± SD of 3-5 experiments) are calculated from the activities of ADA and AK measured in the same experiment. N.D., not determined.

11.3.2. PRPP metabolism

In thymocytes and splenocytes activity of PRPP synthetase was comparable with all species studied (Table 11.4). The activity was also comparable between the species and with the activity in PBL of the same species (chapter 2). When expressed per mg protein some differences were observed. Activity of PRPP synthetase was highest in rat thymocytes and lowest in ovine splenocytes. The activity of PRPP synthetase per mg protein in thymocytes was higher than in splenocytes, except with the horse.

The concentration of PRPP varied considerably between the several preparations of thymocytes of man (Table 11.4). This is probably due to the fact that with human organs it was not possible to standardize storage conditions and the time between death and preparation of cell extracts. With the animals these conditions were more comparable between the individuals of the same species. The concentration of PRPP was lowest in ovine thymocytes and higher in splenocytes than in thymocytes of the same species, except man.

11.4. Discussion

Most enzyme activities of thymus and spleen were measured in

Table 11.3. Activities of ADA, PNP and AK in PBL, thymocytes and splenocytes of various mammalian species

Species		Man	Horse	Pig	Sheep	Rat	Mouse
ADA	PBL	760± 276 (5)	134± 46 (7)	309±119 (9)	2491±1220 (4)	2938± 720 (6)	1162± 344 (3)
	Thymocytes	9597±1667 (3)	220± 60 (3)	4663±980 (3)	9770±1970 (3)	23757±3876 (4)	6687±1014 (3)
	Splenocytes	1590± 398 (3)	411, 297	3911±1074 (3)	2391± 557 (5)	3265± 468 (3)	4000± 985 (3)
PNP	PBL	2450 ± 865 (5)	1561±431 (3)	819±272 (5)	287 ± 143 (6)	4313±1639 (4)	1350 ± 350 (3)
	Thymocytes	2044 ± 519 (3)	1786±593 (3)	1440±122 (3)	183 ± 108 (3)	1494 ± 253 (3)	915 ± 58 (3)
	Splenocytes	2607 ± 497 (4)	1608±766 (3)	648±283 (3)	203 ± 57 (5)	4799 ± 873 (3)	2596±1060 (3)
AK	PBL	61 ± 31 (8)	79 ± 27 (9)	48 ± 22 (7)	167 ± 73 (4)	367 ± 42 (4)	N.D.
	Thymocytes	60 ± 16 (4)	80 ± 39 (3)	23 ± 10 (3)	76 ± 40 (3)	82 ± 49 (4)	99 ± 15 (3)
	Splenocytes	41 ± 19 (3)	43 ± 27 (3)	10 ± 5 (5)	44 ± 12 (3)	424 ± 25 (3)	N.D.

Activities (in nmol/hr per mg protein) are means ± SD for the number of individuals indicated within parentheses. AK was measured in 7000 g supernatant. The PBL were from adult individuals and the thymocytes and splenocytes from individuals of the ages given in Table 11.1.

Table 11.4. Concentration of PRPP and activity of PRPP synthetase in thymocytes and splenocytes of various mammalian species

Species	Age	PRPP concentration		PRPP synthetase in nmol/hr per 10^6 cells		PRPP synthetase in nmol/hr per mg protein	
		Thymocytes	Splenocytes	Thymocytes	Splenocytes	Thymocytes	Splenocytes
Man	p.m.	7.4; 3.1; 33.5	0.8; 4.7; 10.5	3.4 ± 2.0 (3)	2.6 ± 1.6 (4)	208; 216	73; 69
	n.b.	0.9	3.6	4.1	5.8	244	87
Horse	10 mo	1.4	2.5	1.4	4.1	42	94
	12 yr	0.5	N.D.	5.7	2.7	82	6
Pig	6 mo	1.9 ± 0.6 (4)	3.3 ± 2.4 (4)	4.5 ± 1.0 (3)	7.7 ± 2.0 (3)	147 ± 46 (3)	61±40 (3)
Sheep	1 yr	0.66±0.08 (3)	3.9 ± 1.6 (3)	6.8 ± 1.7 (3)	4.8 ± 1.5 (3)	251 ± 22 (3)	12 ± 6 (3)
Rat	40 d	2.2 ± 1.4 (7)	6.7 ± 3.3 (6)	3.5 ± 0.7 (9)	5.2 ± 0.7 (9)	599 ± 230 (9)	114±20 (7)
Mouse	40 d	N.D.	N.D.	3.1 ± 0.5 (3)	4.4 ± 0.7 (5)	195 ± 92 (3)	133±25 (5)

Values are means ± SD for the number of individuals indicated within parentheses. Concentration of PRPP is given in pmol/10^6 cells. Abbreviations are given in Table 11.1.

homogenized tissue without isolation of cells and the activities were expressed per mg protein or per gram wet weight. In the present study we report activities of ADA, PNP, AK, deoxyguanosine kinase and PRPP synthetase and the concentration of PRPP in isolated cells. Dowell et al. (88) and Chechick et al. (59) expressed activities of ADA in human thymus on cell basis and observed higher activities, but Ben-Bassat et al. (22) found a comparable activity. Based on mg protein the activities of ADA reported by several authors (1, 46, 153, 353) are comparable to our values in human thymocytes. The tissues obtained at surgery (1, 58) showed higher ADA activities for human spleen. Hirschhorn et al. (153), Carson et al. (46) and Adams & Harkness (1) reported comparable activities of ADA per mg protein as we did. Carson et al. (46) reported lower values of PNP in thymus and spleen, and comparable values for AK and deoxyguanosine kinase in thymus. The activities of ADA in equine thymocytes and splenocytes reported by Magnuson et al. (223) are comparable with our values. Activities of ADA and PNP in splenocytes and thymocytes of rats agree well with those reported by Barton et al. (15,16). Jackson et al. (182) reported comparable values for ADA and AK and Cartier (52) reported somewhat higher activities in thymocytes of rats. In murine thymocytes a higher activity of ADA per mg protein was found by several authors (345, 372,379), but also lower (43) and comparable activities (295, 371). These differences are probably related to strain variations (371). The activities of ADA per mg protein in murine spleen reported by Tedde et al. (372), Trotta et al. (379) and Willemot et al. (413) are comparable with our values, but Burridge et al. (43) found a lower activity. No literature data for the other species are available.

Pig, horse and sheep are not the only species that have a higher activity of ADA in spleen than in thymus. Trotta et al. (380) measured a higher ADA activity in rabbit splenocytes. With chickens (293) a higher activity of ADA was observed in the spleen and the activity in the bursa was intermediate to the activity in thymus and spleen. In contrast to our results Burridge et al. (43) found a higher activity of ADA in murine spleen than in thymus. In several species the activity of ADA is very high in intestinal tissues (39, 182, 219, 372, 380).

In chapter 10 we demonstrated that the ADA enzyme from rat thymocytes and splenocytes has kinetic properties comparable to that of the low MW type (MW 35 kD, ref. 159). For human and rat spleen this was also previously demonstrated (71, 219, 399). The ADA iso-

zyme in the lymphoid cells of the other species is probably also of this type, since EHNA present in all AK assays almost completely inhibited deamination of adenosine and a high sensitivity to EHNA is a property of the low MW type (294, 296).

Jackson et al. (182) suggested that at a high ADA/AK ratio adenosine will be deaminated, preventing phosphorylation of adenosine. In a comparison of several organs of the rat they found the highest ratio in thymus. Therefore they postulated a correlation between a high proliferation rate and a high ADA/AK ratio. We found with all species a higher ADA/AK ratio in thymocytes than in PBL. Snyder & Lukey (345) showed that with intact murine thymocytes adenosine and deoxyadenosine are predominantly deaminated at concentrations ranging from 0.1-100 μM. With intact equine lymphocytes that have the lowest ADA/AK ratio (Table 11.2) still the same pattern was found (217). The activity of deoxyadenosine kinase is lower than that of AK for all cell types reported (3, 49, 295, chapter 5). For phosphorylation higher Km values have been reported for deoxyadenosine than for adenosine (109, chapter 5). Since ADA showed similar activities with adenosine and deoxyadenosine as substrate in mammalian lymphocytes (chapter 5) and thymocytes of rats of various ages (chapter 10), a high ADA/AK ratio indicates not only that adenosine will be deaminated at physiological concentration in these cells, but also deoxyadenosine. This will prevent accumulation of toxic metabolites of deoxyadenosine.

Deoxyguanosine has been reported to possess selective T-cell toxicity (67). Therefore it will be important to prevent that deoxyguanosine accumulates in the thymus. In ovine thymocytes that have the lowest PNP activity the PNP/deoxyguanosine kinase ratio still is higher than 4. Ovine lymphocytes that have comparable PNP and deoxyguanosine kinase activities as thymocytes predominantly phosphorylyze deoxyguanosine (chapter 8).

It is remarkable that with all species the activity of PRPP synthetase is comparable in thymocytes, splenocytes and PBL (chapter 2) and that its cellular activity is comparable between the species. Activities of all other enzymes that were measured all showed some variations. This could mean that a certain activity of PRPP synthetase is necessary for lymphoid cells, probably because of the important function of PRPP in purine, pyrimidine and pyridine nucletide synthesis. A higher PRPP synthetase activity per mg protein was also found by Weber et al. (410) and Yip et al. (428) for rat and mouse, respectively.

PRPP concentration varied markedly in thymocytes and splenocytes. Its concentration has not been measured before in these cells. Measurement of the PRPP concentration is only meaningful when the organs are rapidly processed and preparation of the organs is standardized. Improper storage conditions can cause cell damage and release of nucleotides and PRPP. The inorganic phosphate concentration can also be altered, which influences PRPP synthesis.

Our studies have shown that considerable differences exist in activities of ADA, PNP, AK and deoxyguanosine kinase in thymocytes, splenocytes and PBL of various mammalian species. They also indicate that thymocytes are more capable than other lymphoid cell types in deaminating adenosine and deoxyadenosine, thus preventing these nucleosides to accumulate and to disturb cell proliferation. The important role of PRPP synthetase for the immune system is indicated by the small variations observed in the activity in this enzyme in the various lymphoid cells of the different mammals, including man.

11.5. Summary

1. Activities of ADA, PNP, AK were measured in splenocytes and thymocytes of newborn children, young horses, pigs, sheep, rats and mice and compared with the activities previously found in PBL. 2. With all species, except horse, the activity of ADA (per 10^6 cells) was higher in thymocytes than in PBL. Activity of ADA was highest in splenocytes of pig and sheep. Activity of ADA was lowest in all lymphoid cells of the horse and only about 10% of the activity in human splenocytes and lymphocytes. 3. With all species, except horse, the activity of PNP was lower in thymocytes than in lymphocytes. Activity of PNP was highest in human lymphocytes and lowest in ovine thymocytes. 4. Activity of AK is comparable in thymocytes of all species and always lower than the ADA activity. In splenocytes of man, horse and pig the activity of AK is comparable to that in thymocytes. 5. Activity of deoxyguanosine kinase was lowest in rat thymocytes and highest in those of man. 6. When enzyme activities are expressed per mg protein, the differences between thymocytes and lymphocytes are less pronounced. 7. Activity of PRPP synthetase per 10^6 cells was comparable in thymocytes, splenocytes and PBL of the same species and between the various species. 8. The concentration of PRPP was lowest in ovine thymocytes and higher in splenocytes than in thymocytes of the same species, except man.

Chapter 12

SURVEY AND SUMMARY

12.1. Purine and pyrimidine metabolism in PBL

12.1.1. The metabolism of PRPP

Synthesis of purine and pyrimidine nucleotides interact at several sites (see 1.4.1). PRPP plays a key role in these interactions since it is a substrate for as well purine de novo as salvage pathways and a substrate for pyrimidine de novo synthesis. The first part of this thesis (chapters 2-4) deals with measurements of the concentrations of PRPP in PBL, the synthesis of PRPP, the regulation of its synthesis and the enzymes in purine and pyrimidine metabolism that use PRPP as a substrate. Furthermore some aspects of PRPP metabolism in PHA-stimulated lymphocytes are reported in chapter 7. It was demonstrated that previously described methods for estimating the concentration of PRPP and the activity of PRPP synthetase in erythrocytes (see thesis Tax) could also be used for lymphocytes (chapter 2) and lymphoid tissues (chapter 11).

The concentration of PRPP and the activity of PRPP synthetase were comparable in lymphocytes of man, horse, pig, sheep and cattle (chapter 2). In chapter 3 the kinetic properties of PRPP synthetase from man and horse were described. The enzyme showed biphasic kinetics for ATP, with a low Km of about 5 µM with both species. For ribose 5-P Km values of about 25 µM were found. Both substrates of PRPP showed substrate inhibition. Of 35 compounds tested at 5 mM concentration adenine and guanine nucleotides markedly inhibited the PRPP synthetase from equine lymphocytes. SAH also strongly inhibited the enzyme, but inhibition of pyrimidine nucleotides was lower. AMP, dAMP, ADP, dADP and GMP also inhibited the enzyme at 0.1 mM concentration at 30 µM ATP but not at 30 µM ribose 5-P. Inhibition by AMP was competitive with a Ki value of 7 µM. ADP, dADP and dAMP showed other types of inhibition with higher Ki values. Since these nucleotides can accumulate in ADA deficiency inhibition of PRPP synthetase can play a role in the impairment of cell proliferation that is found with these patients.

Activities of enzymes that utilize PRPP as a substrate show

large variations among the various species (chapter 2). The activity of the purine salvage enzyme HPRT is highest in equine lymphocytes, lowest in those of cattle and intermediate in those of man, pig and sheep. With all species the activity of APRT is lower than that of HPRT, except man. The activity of APRT is comparable in lymphocytes of man and horse and lower in those of cattle, sheep and pig. With all species the activity of OPRT was lower than that of HPRT. The OPRT activity was lowest in lymphocytes of horse and highest in those of pig. Activity of ODC was always higher than that of OPRT and it was coordinate with OPRT with all species. Porcine lymphocytes were used to study the effects of several metabolites on the activities of OPRT and ODC (chapter 4). Only CMP and UMP inhibited ODC. Adenine and guanine nucleosides, guanine nucleotides and several pyrimidine nucleotides inhibited OPRT at 5 mM concentration.

12.1.2. Metabolism of nucleosides and nucleotides

Several differences were observed between the various species in the metabolism of uridine (chapter 4). Activity of uridine kinase was very low in lymphocytes of sheep and those of several pigs. In lymphocytes of other pigs a significantly higher activity was found. Activity of uridine kinase was intermediate in lymphocytes of man, horse and cattle. The activity of uridine phosphorylase was higher than that of uridine kinase of the same species, except horse. Activity of uridine phosphorylase was highest in lymphocytes of man and pig. Breakdown of UMP, catalyzed by a 5'-nucleotidase and a non-specific phosphatase (chapter 5) was comparable with lymphocytes of horse, man, pig and sheep. In man and pig the activity was comparable with that of uridine phosphorylase.

Chapter 5 further deals with the various enzymes that play a role in the metabolism of adenosine and deoxyadenosine. The activity of ADA in lymphocytes of man is comparable with that in lymphocytes of 8 other mammalian species (see also chapter 11), but its activity is very low in lymphocytes of horse and pig and about 10% of that in human lymphocytes. There is no general correlation between the activity of ADA in lymphocytes and erythrocytes, since porcine lymphocytes have an activity comparable to that of human erythrocytes and higher than that in erythrocytes of sheep, goat and cattle. No ADA activity was detectable in equine erythrocytes.

In Fig. 12.1 the metabolism of adenosine in human, equine and

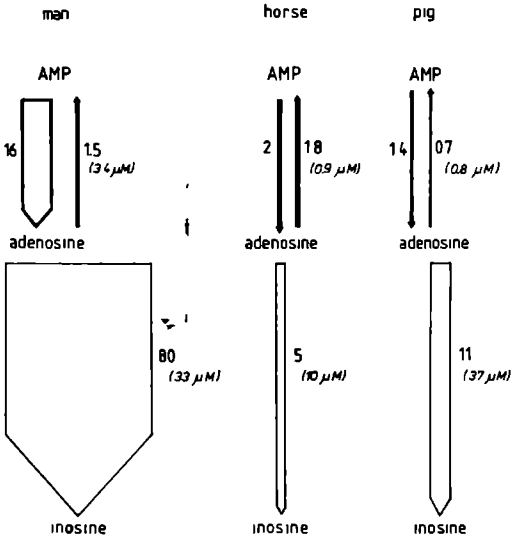


Fig. 12.1. Metabolism of adenosine in lymphocytes of man, horse and pig. Values represent enzyme activities in nmol/hr per 10^6 cells. Km values are given within parentheses.

porcine lymphocytes is summarized. Since the concentration of AMP in these lymphocytes is comparably low (chapter 9) and the activity of AMP nucleotidase is lower in equine and porcine lymphocytes, the contribution of AMP to the adenosine pool will be lower in these cells than in human lymphocytes. With all species activity of AK was lower than that of ADA, but the Km values for phosphorylation are also lower. Since the animal lymphocytes have a lower Km value for phosphorylation of adenosine than human lymphocytes, adenosine can more easily be phosphorylated in the animal lymphocytes. The low ADA activity in equine lymphocytes can more efficiently be used, because of the low Km value. With porcine and human lymphocytes AMP can also be deaminated. This seems not probably in lymphocytes of horse and sheep, because they have a very low activity of AMP deaminase. The breakdown of AMP and IMP in animal lymphocytes is not only catalyzed by a 5'-nucleotidase but also by a non-specific phosphatase. With intact animal lymphocytes not only an ecto-5'-nucleotidase but also an ecto-non-specific phosphatase was found. With human lymphocytes these non-specific phosphatase activities were low, but the activities of total and of specific ecto-5'-nucleotidase were markedly higher.

The rate of deamination of deoxyadenosine is comparable to that of adenosine in all species. Deoxyadenosine deamination capacity is much higher in human than in equine and porcine lymphocytes, but with human and porcine lymphocytes higher Km values were observed. Phosphorylation rates of deoxyadenosine were lower than that of adenosine and higher Km values were observed (about 500 µM with equine and porcine lymphocytes and 76 µM with human lymphocytes). This means

that with lymphocytes of all species deoxyadenosine will predominantly deaminated under physiological conditions. Adenosine inhibited deoxyadenosine phosphorylation at equimolar concentrations with equine and porcine lymphocytes and deoxycytidine with human and porcine lymphocytes. This gives further evidence that various kinases are involved in deoxyadenosine phosphorylation (see section 1.2.1).

The activity of SAH hydrolase in the synthetic direction was much lower than that of ADA in extracts of lymphocytes of all species. Deoxyadenosine was able to inactivate the enzyme with all species. In vivo this inactivation can result in an accumulation of SAH, that can inhibit methylation reactions (see section 1.8).

The activity of PNP was highest in human lymphocytes (chapter 5), lowest in those of sheep and goat (about 5% of that in man) and intermediate in those of the other species. Like with ADA there was no correlation between activities in lymphocytes and erythrocytes.

In chapter 6 the metabolism of the substrates of PNP is further investigated in lymphocytes of man and sheep and in thymocytes of rat that also have a low PNP activity. The activity of PNP was higher with inosine than with guanosine and deoxyguanosine as substrates in lymphocytes of man. With ovine lymphocytes a comparably low activity is found with these three substrates. Km values for deoxyguanosine were comparable (about 100 µM) in lymphocytes of both species and rat thymocytes. Km values for inosine and guanosine in ovine lymphocytes (about 50 µM) were lower than in human lymphocytes (about 100 µM). The rate of phosphorylation of deoxyguanosine and guanosine was much lower than the phosphorylysis rate in extracts of human and ovine lymphocytes and of rat thymocytes. No guanosine phosphorylating activity was detectable in extracts of rat thymocytes. The Km value for deoxyguanosine kinase activity was highest with human lymphocytes (about 1.6 mM), lowest with ovine lymphocytes (about 0.1 mM) and intermediate in rat thymocytes. With intact cells the rate of phosphorylysis of deoxyguanosine, guanosine and inosine was highest with human lymphocytes. At low deoxyguanosine concentration (20 µM) phosphorylation was comparable to phosphorylysis with ovine lymphocytes. At higher concentrations the rate of phosphorylysis exceeds that of phosphorylation, as was also found with the other species at all concentrations of deoxyguanosine. Guanosine and inosine were also incorporated into nucleotides and nucleic acids by intact human and ovine lymphocytes and rat thymocytes. The incorporation of inosine was almost completely inhibited by hypoxanthine, but that of deoxyguanosine and guanosine by about 50%.

The difference present in purine and pyrimidine metabolism between the various species are not reflected in the concentrations of the various nucleotides (chapter 9). ATP appeared to be the most predominant nucleotide in lymphocytes of man, horse, pig and sheep and in rat thymocytes. Its concentration was about 850 pmol/10^6 cells in human and equine lymphocytes, higher in those of pig and lower in those of sheep. The concentration of GTP was comparable in human, equine and porcine lymphocytes (about 175 pmol/10^6 cells), but lower in ovine lymphocytes. UTP and CTP concentrations were lower and comparable in the four species; other nucleotides, except ADP (high in porcine lymphocytes), did not show marked species related differences. The energy charge for purine and cytosine ribonucleotides did not show significant differences and varied between 0.83 and 0.88 in the lymphocytes of the various species; the energy charge of the uracil ribonucleotides was slightly higher with all species.

12.2. Purine metabolism in lymphoid tissues

Chapters 10 and 11 deal with measurements of several enzymes in purine metabolism of cells from thymus and spleen. With rat the activities of ADA and PNP were determined in tissues obtained from rats of 0-423 days old (chapter 10). ADA activity per cell is highest in thymocytes of newborn rats and decreases during postnatal development, while in splenocytes ADA activity increases. No significant age-dependency is found with PNP in thymocytes and splenocytes. ADA activity is higher than that of PNP in thymocytes during whole life, while in splenocytes the PNP activity is higher. No age-dependency for AK was found with rats of 13-60 days.

With thymocytes the ADA activity with deoxyadenosine is higher than with adenosine. No significant differences were found in Km values of both nucleosides in thymocytes of 3- and 40-days old rats (30-40 µM). The high ADA activity in thymocytes of newborn rats can result in a good protection against adenosine and deoxyadenosine toxicity.

The activities of ADA, PNP and AK were also measured in splenocytes and thymocytes of newborn children, young horses, pigs, sheep and mice. With all species, except horse, ADA activity (per 10^6 cells) was higher in thymocytes than in PBL. The ADA activity was highest in splenocytes of sheep and pig, and lowest in all lymphoid cells of horse. With man, horse, rat and mouse the activity of ADA was comparable in their splenocytes and PBL. The activity of AK is

comparable in thymocytes of all species and always lower than that of ADA. In splenocytes of man, horse and pig the activity of AK is comparable to that in thymocytes. The high ADA/AK ratio suggests that in all lymphoid tissues adenosine will predominantly deaminated at physiological concentrations.

The activity of PNP was highest in human PBL and lowest in PBL and thymocytes of sheep. With all species, except horse, the PNP activity was lower in thymocytes than in PBL. The activity of deoxyguanosine kinase was lowest in thymocytes of the rat and highest in those of man. Due to the high PNP/deoxyguanosine kinase ratio deoxyguanosine will be mainly phosphorylysed (see also chapter 9).

When the activities were expressed per mg protein the differences between thymocytes and PBL are less pronounced.

The activity of PRPP synthetase per 10^6 cells is comparable in thymocytes, splenocytes and PBL of the same species and between the various species. This could mean that a certain activity of PRPP synthetase is necessary for lymphoid cells, probably because of the important function of PRPP in purine, pyrimidine and pyridine nucleotide synthesis. The PRPP concentration varies between the several species and with man also between the various preparations of thymocytes and splenocytes. The latter is probably due to the fact that not all conditions could be standardized. The lowest PRPP concentration was found in ovine thymocytes. With all species, except man, the concentration was higher in splenocytes than in thymocytes of the same species.

12.3. Mitogenic stimulation of mammalian lymphocytes

12.3.1. Optimalization of methods

In chapter 6 culture conditions are described for PHA-stimulation of lymphocytes of horse, pig, sheep and man in MEMS medium supplemented with horse serum. Cells could be cultured in a volume of 0.2 ml in microtitertek plates but also in a volume of 1 ml in silanized glass tubes (see chapters 3,4 and 7). Rat thymocytes responded badly to PHA and Con-A in MEMS medium (chapter 8), but in RPMI 1640 medium supplemented with horse serum (10%) a good response to Con-A was obtained. Since we wanted to study the effects of several metabolites (chapters 7-9) on thymidine and uridine incorporation as parameters of mitogenic stimulation, we optimized the concentrations of these nucleosides to prevent inhibition of their uptake by

the metabolites.

12.3.2. PRPP and pyrimidine metabolism in PHA-stimulated lymphocytes

Several of the parameters tested at PHA-stimulation of mammalian lymphocytes (chapters 3 and 4) are summarized in Table 12.1. Although the activity of PRPP synthetase does not increase at PHA-stimulation, a higher concentration of PRPP is found in PHA-stimulated lymphocytes. It has to be established if this increase in PRPP concentration is due to the higher rate of the pentose phosphate pathway that was found by other investigators at PHA-stimulation (see section 1.5). The PRPP concentration in non-stimulated lymphocytes also increases at culturing but to a lower extent than in PHA-stimmulated cells.

The capacity of the PRPP metabolizing enzyme OPRT decreases at PHA-stimulation of porcine lymphocytes. In vivo its activity can be relatively higher at PHA-stimulation, due to the higher availability of PRPP. The salvage pathway probably plays a more important role in pyrimidine nucleotide supply of peripheral and PHA-stimulated lymphocytes of horse, but in those of pig the de novo pathway probably also has an important role.

Table 12.1. Effect of PHA-stimulation on PRPP and pyrimidine metabolism

Parameter	Species	Effect
PRPP synthetase	Man	No change
	Horse	No change
	Pig	No change
PRPP concentration	Man	Increase (3-8 fold)
	Horse	Increase (2-4 fold)
	Pig	Increase (5-12 fold)
OPRT and ODC	Pig	Decrease
Uridine kinase	Horse	Increase (2-4 fold)
	Pig	Increase (2-10 fold) or decrease
Uridine phosphorylase	Horse	Increase (2-7 fold)
	Pig	Increase (2-12 fold)

12.3.3. Effect of nucleosides on mitogenic stimulation of mammalian lymphocytes

In chapters 7-9 the effects of various nucleosides on mitogenic stimulation of human, equine, porcine and ovine lymphocytes and rat thymocytes are reported. Some of the results are summarized in Table 12.2. Adenosine is the most potent inhibitor of thymidine incorporation at 51 hr with all species studied, except pig. In porcine lymphocytes adenosine and deoxyadenosine caused a considerable elevation of the incorporation. Uridine incorporation is equally affected as thymidine incorporation, but the effects on leucine incorporation, as well stimulation as inhibition are lower. The effects were time-dependent. After 75 hr inhibition of thymidine incorporation of equine PHA-stimulated lymphocytes and stimulation of thymidine incorporation of porcine PHA-stimulated lymphocytes were lower. This is probably due to the rapid breakdown of adenosine and deoxyadenosine, as was established with HPLC. Addition of EHNA, an inhibitior of ADA, in combination with 25 μM nucleoside, resulted in a higher inhibition of thymidine incorporation with equine and human lymphocytes. With equine lymphocytes the inhibition by adenosine plus EHNA was higher than that of deoxyadenosine plus EHNA, but with human lymphocytes the combination deoxyadenosine plus EHNA showed a higher inhibition.

Table 12.2. Effect of purine nucleosides and hypoxanthine on thymidine incorporation of mitogen-stimulated mammalian lymphoid cells

Nucleoside	Lymphocytes				Thymocytes
	Man	Horse	Pig	Sheep	Rat
Adenosine	21	16	532	-	-
Deoxyadenosine	94	61	459	-	-
Inosine	117	-	345	135	91
Deoxyinosine	114	-	-	65	107
Hypoxanthine	-	-	490	-	-
Guanosine	123	-	-	114	88
Deoxyguanosine	73	-	-	99	67

Values represent the relative incorporation (in % of the control value) at 51 hr. All compounds were tested with lymphocytes at 100 μM, but with rat thymocytes at 50 μM.

With porcine lymphocytes, addition of EHNA reverted the stimulation by deoxyadenosine of thymidine and uridine incorporation in a marked inhibition, but the combination adenosine plus EHNA still stimulated incorporation. Deoxycytidine was not able to prevent the inhibition by (deoxy)adenosine plus EHNA completely; with equine lymphocytes even a higher inhibition was found in some cases. The effects of adenosine and deoxyadenosine were not additional as was established with equine lymphocytes. Only in the presence of homocysteine the effect of adenosine and deoxyadenosine was higher than adenosine plus homocysteine or deoxyadenosine plus homocysteine.

The effects of the degradation products of adenosine and deoxyadenosine were investigated to get more insight in the mechanism of the stimulation of thymidine and uridine incorporation of porcine lymphocytes. Inosine and hypoxanthine also stimulated the incorporation. Probably hypoxanthine is converted to IMP and subsequently to AMP and/or GMP. The simultaneous suppression of the PHA-stimulated increase in PRPP concentration by adenosine found in porcine lymphocytes may be related to this conversion, but also to inhibition of PRPP synthesis by AMP. The decrease of the PRPP concentration by adenosine with equine lymphocytes can only be due to inhibition of PRPP synthesis by AMP.

Adenosine inhibited the thymidine and uridine incorporation more than the other purine nucleosides with human lymphocytes. Inosine at 0.5 mM did not inhibit thymidine incorporation of human and ovine lymphocytes and slightly inhibited thymidine incorporation of Con-A stimulated rat thymocytes. Deoxyinosine showed some inhibition at 0.5 mM with all cells studied, guanosine only with human and ovine lymphocytes but not with rat thymocytes. Deoxyguanosine inhibited thymidine incorporation of ovine PHA-stimulated lymphocytes only at 0.5 mM but with human lymphocytes and rat thymocytes also at 50 μM. It appears that of the substrates of PNP deoxyguanosine is most toxic to lymphoid cells.

To get more insight in the mechanism of the effects of adenosine and deoxyadenosine the concentrations of ATP, dATP and TTP were measured in PHA-stimulated lymphocytes of man, horse and pig that were treated with these nucleosides with or without EHNA (chapter 9). Concentrations of ATP and TTP in treated cells showed some differences with the concentrations in PHA-stimulated cells without additions, but not as marked as those of dATP. The concentration of dATP increased in all cultures treated with deoxyadenosine and EHNA, with porcine and equine lymphocytes about 10-fold and with

human lymphocytes about 3-fold. All these cultures also showed a marked inhibition of thymidine and uridine incorporation. These results seem to support the hypothesis that inhibition of cell proliferation is mediated by dATP (see section 1.7). However, in porcine PHA-stimulated lymphocytes that were incubated with 100 μM deoxyadenosine as well the thymidine and uridine incorporation increased as the dATP concentration, even 5-fold. This means that a high concentration of dATP is not necessarily associated with inhibition of cell proliferation. Therefore the toxic effects of deoxyadenosine may also be mediated by other mechanisms than inhibition of ribonucleotide reductase. Inhibition of pyrimidine nucleotide synthesis is not excluded, but the effects of deoxyadenosine on synthesis of PRPP and energy metabolism need further investigation.

Our results have given additional evidence that ADA and PNP play an important role in the lymphoid cells. The immune disorders caused by deficiency of these enzymes can not be explained by one mechanism and may be related to disturbance of cell differentiation and maturation or cell function. The increased interest in purine and pyrimidine metabolism of lymphoid cells caused by the discovery of these enzyme deficiencies has not resulted in a specific therapy for these disorders. However, the increased insight may be applied to use purine and pyrimidine nucleosides and nucleoside analogs or inhibitors of purine metabolism for specific manipulation of the immune system as is demanded in transplantation therapy and cancer chemotherapy. ADA inhibitors like deoxycoformycin can be used for selective suppression of uncontrolled proliferation of leukemic cells and to increase the potency of cytostatic nucleoside analogs. Since the substrates of PNP primarily affect thymus-derived cells, inhibition of the ontogenesis of these cells could be achieved by specific inhibition of PNP.

Biochemical methods have been developed that make it possible to study the effects of purine and pyrimidine metabolites and antimetabolites in vitro. From our comparative experiments the significance of several metabolic routes has been underlined. The insight that has been obtained in the metabolism of PRPP can be used to manipulate actions of drugs that depend on the availability of PRPP to be converted to their active form. Since mammalian lymphoid cells do not show pronounced differences in the metabolism of PRPP animal cells form a good system for further investigation of the pharmacological effects of drugs that interfere with the availability of PRPP.

Purine en pyrimidine metabolisme in lymfoide cellen van de mens en enkele andere zoogdier species

Purine en pyrimidine nucleotides zijn essentiële onderdelen van RNA en DNA en van een aantal belangrijke coënzymen. Om de benodigde concentraties van de verschillende nucleotides te handhaven, moeten ze in voldoende hoeveelheden aangemaakt worden. Dit kan bij zowel het purine als het pyrimidine metabolisme gebeuren uit eenvoudige verbindingen zoals bicarbonaat en aminozuren via de zogenaamde 'de novo' routes. Energetisch gezien voordeliger zijn de 'salvage' of reutilizatie routes die nucleosides en basen die ontstaan uit de afbraak van nucleïnezuren, opnieuw gebruiken voor de synthese van nucleotides. Het purine en pyrimidine metabolisme zijn geen afzonderlijke eenheden, maar houden op vele plaatsen verband met elkaar en ook met het metabolisme van aminozuren en koolhydraten. Een stof die een belangrijke rol speelt in deze interacties is het fosforibosylpyrofosfaat (PRPP).

In hoofdstuk 1 wordt uitgebreid ingegaan op het huidige inzicht omtrent de regulatie van het purine en het pyrimidine metabolisme, in het bijzonder in de perifere lymfocyt en de met mitogeen gestimuleerde lymfocyt. Enkele aspecten van het immuunsysteem worden besproken, omdat storingen in het purine metabolisme, zoals deficiënties van de enzymen adenosine deaminase (ADA) en purine nucleoside fosforylase (PNP) in de lymfocyt, geassocieerd bleken te zijn met storingen in het immuunsysteem. De metabole basis van de mechanismen die hiertoe zouden kunnen leiden, vormden de aanleiding tot dit onderzoek. Vergelijkend onderzoek aan lymfoïde cellen van de mens, paard, varken, schaap, rat en enkele andere zoogdieren leverde een beter inzicht in verschillende aspecten van het purine en het pyrimidine èn het PRPP metabolisme van deze cellen. Enkele aspecten die hierbij bijzonder de aandacht hadden, waren een vergelijking van perifere en gestimuleerde lymfocyten; een vergelijking van perifere lymfocyten met thymus en milt cellen; de relatie tussen purine metabolisme en de ontwikkeling van lymfoïde cellen en de effecten van purine (deoxy)nucleosides op de mitogene stimulering van lymfoïde cellen. De resultaten toonden aan dat er waarschijnlijk meer biochemische verklaringen zijn voor de relatie tussen de enzymdeficiënties en de immuundeficiënties. Het verkregen inzicht in de regulatie van purine, pyrimidine en PRPP metabolisme kan een bijdrage leveren tot een

meer specifieke beïnvloeding van het immuunsysteem zoals dat vereist is bij chemotherapie van leukemieën en lymfomen en immuunsuppressie bij transplantatie. De ontwikkeling van lymfoïde cellen kan selectief geremd worden door bepaalde purine (deoxy)nucleosides of analogen en/of remmers van ADA of PNP. Het verkregen inzicht in het metabolisme van PRPP kan van nut zijn bij het gebruik van antimetabolieten die met PRPP in hun actieve vorm omgezet worden.

In hoofdstuk 2 wordt gerapporteerd over de aktiviteiten van de enzymen in het purine en pyrimidine metabolisme die PRPP als substraat gebruiken, de fosforibosyltransferases. De purine fosforibosyltransferases, het adenine fosforibosyltransferase en het hypoxanthine-guanine fosforibosyltransferase, hadden relatief de hoogste aktiviteiten in lymfocyten van paard en mens, terwijl de aktiviteiten van de pyrimidine enzymen, orootzuur fosforibosyltransferase en orotidylaat decarboxylase erg laag waren in lymfocyten van het paard en hoog in die van het varken. De aktiviteit van het PRPP synthetase en de concentratie van PRPP waren vergelijkbaar in lymfocyten van het paard, varken, schaap, rund en de mens.

In hoofdstuk 3 wordt uitgebreid aandacht besteed aan de kinetiek van het PRPP synthetase uit humane en paarde lymfocyten. Bij beide species werd een bifasische kinetiek met ATP gevonden, terwijl beide substraten, ATP en ribose 5-fosfaat een substraat remming vertoonden bij relatief hoge concentraties. AMP, deoxyAMP, ADP en deoxyADP bleken de aktiviteit van het PRPP synthetase aanzienlijk te remmen. De remming door guanine en pyrimidine nucleotides was relatief lager. Remming van de PRPP synthese zou een rol kunnen spelen bij de gestoorde proliferatie van cellen zoals die gevonden is bij ADA en PNP deficiënties.

In hoofdstuk 3 wordt het effect van stimulatie door phytohemagglutinine (PHA) van lymfocyten op de PRPP synthese beschreven. Hoewel PHA in drie-daagse cultures van humane, paarde en varkens lymfocyten nauwelijks effect heeft op de aktivieit van het PRPP synthetase, blijkt de PRPP concentratie 3-11 keer verhoogd te zijn. Een verklaring hiervoor is niet gevonden.

In hoofdstuk 4 worden de aktiviteiten van uridine kinase en uridine fosforylase gerapporteerd in de lymfocyten van enkele zoogdierspecies, waaronder de mens. Bovendien wordt het effect van PHA beschreven op de aktiviteit van deze enzymen en op de aktiviteit van het orootzuur fosforibosyltransferase en het orotidylaat decarboxylase. Tenslotte wordt de invloed van enkele pyrimidines en purines op de aktiviteiten van het orootzuur fosforibosyltransferase en het

orotidylaat decarboxylase uit varkens lymfocyten beschreven.

In hoofdstuk 5 worden de capaciteiten en de kinetiek beschreven van een aantal enzymen in lymfocyten van mens, paard en varken die een rol spelen bij de synthese en de afbraak van adenosine en deoxyadenosine. Bij vergelijkende metingen aan lymfocyten van 10 zoogdier species bleek dat de aktiviteit van ADA in lymfocyten van paard en varken ongeveer 10% was van die in humane lymfocyten en vergelijkbaar met die bij kinderen met ADA deficiëntie. Verder bleek de aktiviteit van PNP erg laag te zijn in lymfocyten van schaap en geit. De aktiviteit van adenosine kinase is veel lager dan die van adenosine deaminase ook bij lymfocyten van paard en varken; de Km waardes zijn echter ook lager zodat bij een fysiologische adenosine concentratie dit nucleoside voornamelijk gefosforyleerd zal worden. De Km waardes voor fosforylering van deoxyadenosine zijn veel hoger dan voor deaminering, terwijl de aktiviteiten lager liggen; deoxyadenosine zal dus vooral gedeamineerd worden.

Adenosine kan ontstaan uit AMP in een reactie die bij lymfocyten van paard, varken en schaap gekatalyseerd wordt door het 5'-nucleotidase en een non-specifiek fosfatase, bij de mens echter alleen door het 5'-nucleotidase. De defosforylering geschiedt met dezelfde snelheid bij lymfocyten van paard en varken, en is hoger bij die van schaap en mens. De vorming van IMP uit AMP door AMP deaminase is in lymfocyten niet belangrijk. AMP en IMP bleken ook door een ecto-5'-nucleotidase en door een ecto-non-specifiek fosfatase in paarde en varkens lymfocyten afgebroken te kunnen worden. De aktiviteit van het S-adenosyl-homocysteïne hydrolase werd bepaald en bleek geïnaktiveerd te kunnen worden door incubatie met deoxyadenosine. In vivo kan dit leiden tot remming van methylerings reacties.

In hoofdstuk 6 is de optimalisering van cultuurcondities voor stimulering met PHA van lymfocyten van mens, paard, varken en schaap beschreven. De lymfocyten van deze species bleken in hetzelfde medium gesupplementeerd met paardeserum gekweekt te kunnen worden. De condities werden geoptimalizeerd voor het meten van de thymidine incorporatie en gebruikt voor de stimulatie experimenten beschreven in de hoofdstukken 3,4,7,8 en 9.

In hoofdstuk 7 zijn de effecten beschreven van adenosine, deoxyadenosine en homocysteïne met en zonder EHNA, een remmer van ADA, op de thymidine, uridine en leucine incorporatie van PHA-gestimuleerde lymfocyten van mens, paard en varken. De effecten op de thymidine en uridine incorporatie waren vergelijkbaar, terwijl die op de leucine incorporatie lager waren. Adenosine bleek de thymidine incorpo-

ratie van paarde en humane lymfocyten sterker te remmen dan deoxyadenosine, terwijl beide nucleosides de thymidine incorporatie van varkenslymfocyten meer dan 5-voudig verhoogden. Toevoeging van EHNA versterkte de remmende effecten van adenosine en deoxyadenosine bij paarde en humane lymfocyten, terwijl met varkens lymfocyten de stimulering van de thymidine incorporatie veranderde in een remming. De effecten van adenosine en deoxyadenosine waren niet additief. Adenosine verlaagde bij zowel paarde als varkens lymfocyten de verhoging van de PRPP concentratie die normaal bij PHA-stimulatie gevonden werd.

In hoofdstuk 8 wordt het metabolisme beschreven van de substraten van PNP in lymfocyten van mens en schaap en ratte thymocyten en hun effecten op mitogene stimulatie. Inosine bleek een beter substraat voor PNP dan guanosine en deoxyguanosine voor lymfocyten van de mens zowel met cel extracten als met intacte cellen. In cellen en extracten van alle drie species kon deoxyguanosine gefosforyleerd worden, terwijl fosforylering van guanosine alleen gevonden werd met humane en schape lymfocyten. De fosforylering van deoxyguanosine en guanosine in intacte cellen bleek gedeeltelijk te lopen via de fosforylyse en vervolgens omzetting van de base met PRPP tot nucleotide. De fosforylering van inosine bleek geheel via deze route lopen. Inosine en deoxyinosine bleken geen respectievelijk weinig effect te hebben op de PHA-stimulering van humane en schape lymfocyten en op de Concanavaline-A stimulering van ratte thymocyten. Guanosine bleek de thymidine incorporatie van Concanavaline-A gestimuleerde ratte thymocyten niet te remmen terwijl een relatief lage deoxyguanosine concentratie al een remmend effect had. Perifere lymfocyten van mens en schaap waren minder gevoelig voor deoxyguanosine, terwijl guanosine hier wel een remmend effect vertoonde.

In hoofdstuk 9 zijn de concentraties gegeven van de purine en pyrimidine ribonucleotides en van TTP en deoxyATP in perifere lymfocyten van paard, varken, schaap en mens. De concentratie van ATP was het hoogst bij alle species, en in dezelfde orde van grootte bij paard, mens en varken en lager bij het schaap. De concentraties van TTP en deoxyATP waren erg laag. De concentraties van TTP, deoxyATP en ATP werden ook gemeten in gekweekte lymfocyten (gestimuleerd met PHA en niet gestimuleerd) met en zonder adenosine of deoxyadenosine. Er werden kleine veranderingen gevonden in de concentraties van ATP en TTP in de aanwezigheid van adenosine of deoxyadenosine, maar de concentratie van deoxyATP was aanzienlijk verhoogd in PHA-gestimuleerde lymfocyten van paard, varken en mens in de aanwezigheid van

deoxyadenosine en EHNA. Bij varkens lymfocyten werd met deoxyadenosine alleen ook een verhoging gevonden van de deoxyATP concentratie die niet geassocieerd was met een remming van de thymidine incorporatie, dit in tegenstelling tot bij lymfocyten van paard en mens waar een verhoogde deoxyATP concentratie altijd geassocieerd was met een remming van de thymidine incorporatie.

In hoofdstuk 10 zijn de aktiviteiten gerapporteerd van ADA en PNP in geïsoleerde thymus en milt cellen van ratten van 0-423 dagen oud. De aktiviteit van ADA bleek leeftijd gebonden te zijn en af te nemen in thymocyten bij postnatale ontwikkeling. De PNP aktiviteit in thymus cellen was altijd lager dan de ADA aktivieit, het omgekeerde was het geval in de milt cellen. Er was geen verschil in de aktiviteit van het adenosine kinase in thymus cellen van ratten van verschillende leeftijden. Ook de K_m waarden voor adenosine en deoxyadenosine in ratten van 3 en 40 dagen oud vertoonden geen verschillen.

In hoofdstuk 11 zijn de metingen uit hoofdstuk 10 uitgebreid met die in thymus en milt cellen van pasgeboren kinderen, veulens, jonge varkens, schapen en muizen. De aktiviteit van ADA bleek het hoogst te zijn in miltcellen van schaap en varken en het laagst in thymus cellen, milt cellen en perifere lymfocyten van het paard. Bij de andere species was de aktiviteit van ADA hoger in thymus cellen dan in milt cellen. Er werden geen grote verschillen gevonden in de aktiviteiten van adenosine kinase tussen de verschillende species in de diverse typen cellen. De aktiviteit van ADA was altijd veel hoger dan die van adenosine kinase. De PNP aktiviteit was het hoogst in perifere lymfocyten van de mens en het laagst in de thymus cellen van het schaap. De aktivieit van PRPP synthetase was vergelijkbaar in thymus cellen, milt cellen en perifere lymfocyten van alle species en ook tussen de species onderling. De concentratie van PRPP vertoonde enige verschillen tussen de diverse cel types van de verschillende species.

In hoofdstuk 12 tenslotte is getracht in een algemeen overzicht verbanden te leggen tussen de resultaten die in de verschillende hoofdstukken gerapporteerd zijn.

1. Adams A & Harkness RA (1976) Clin. Exp. Immunol. 26, 647-649
2. Agarwal RP & Parks RE (1978) Methods Enzymol. 51, 502-507
3. Agarwal RP (1981) Comp. Biochem. Physiol. 70B, 595-599
4. Akasaka TL, Olds-Arroyo L & Miller JJ (1979) Experientia 35, 673-674
5. Allison AC (1976) TIBS 1, 36-38
6. Allsop J & Watts RWE (1978) J. Molec. Med. 3, 105-110
7. Andres CM & Fox IH (1979) J. Biol. Chem. 254, 11388-11393
8. Arch JRS & Newsholme EA (1978) Biochem. J. 174, 965-977
9. Arch JRS & Newsholme EA (1978) In: Essays Biochem. (Eds. Campbell PN & Aldridge WN) Vol. 14, pp. 82-123. Academic Press, London
10. Ashihara H (1977) Z. Pflanzenphysiol. 85, 383-392
11. Atkinson DE & Fall L (1967) J. Biol. Chem. 242, 3241-3242
12. Bagnara AS, Letter AA & Henderson JF (1974) Biochim. Biophys. Acta 374,259-270
13. Ballet JJ, Insel R, Merler & E Rosen FS (1976) J. Exp. Med. 143, 1271-1275
14. Barankiewicz J, Battell ML & Henderson JF (1977) Can. J. Biochem. 55, 854-840
15. Barton RW & Goldschneider I (1979) Mol. Cell. Biochem. 28, 135-147
16. Barton RW, Martiniuk F, Hirschhorn R & Goldschneider I (1980) Cell. Immunol. 49, 208-214.
17. Becher HJ, Weber M & Löhr GW (1978) Klin. Wschr. 56, 275-283
18. Becher HJ & Löhr GW (1979) Klin. Wschr. 57, 1109-1115
19. Becker MA, Meyer MJ & Seegmiller JE (1973) Am. J. Med. 55, 232-242
20. Becker MA, Kostel PJ & Meyer LJ (1975) J. Biol. Chem. 250, 6822-6830
21. Becker MA, Raivio KO & Seegmiller JE (1979) In: Advances in enzymology and related areas of molecular biology (Ed. A. Meister), Vol. 29, pp. 281-306. John Wiley & Sons, Inc, New York
22. Ben-Bassat J, Simoni F, Holtzmann F & Ramot B (1979) Israel J. Med. Sci. 15, 925-927
23. Benke PJ & Dittmar D (1976) Ped. Res. 10, 642-646
24. Bennett LL, Hill DL & Allan PW (1978) Biochem. Pharmacol. 27, 83-87
25. Biggar WD, Giblett ER, Ozere RL & Grover BD (1978) J. Pediat. 92, 354-357
26. Blatt J, Reaman G & Poplack DG (1980) N. Engl. J. Med. 303, 918-922
27. Block LH, Lüthy R & Siegenthaler W (1980) Klin. Wschr. 58, 1271-1281
28. Blomquist CH, Larson KE & Taddeini L (1976) Exp. Cell Res. 100, 447-450
29. Bluestein HG, Willis RC, Thompson LF et al. (1978) Trans. Assoc. Am. Phys. 92, 394-402
30. Board PG & Smith JE (1977) Biochem. Gen. 15, 439-444
31. Bollum FJ & Goldschneider I (1980) In: Membranes, receptors and the immune response. pp. 189-202. Alan. R. Riss, Inc., New York
32. Bollum FJ (1981) TIBS 6, 41-43
33. Borgers M, Verhaegen H, De Brabander M et al. (1977) J. Immunochem. Meth. 16, 101-110
34. Borgers M & Thone F (1978) Histochem. J. 10, 721-730
35. Borkowsky W, Gershon L, Shenkman L & Hirschhorn R (1980) Pediat. Res. 14, 885-889
36. Boss GR, Thompson LF, Spiegelberg HL et al. (1979) Trans. Ass. Am. Phys. xcii, 309-315
37. Boss GR, Thompson LF, Spiegelberg HL et al. (1980) J. Immunol. 125, 679-682

38. Böyum A (1968) Scand. J. Clin. Lab. Invest. 21 (suppl. 97), 77-89
39. Brady TG & O'Donovan CJ (1965) Comp. Biochem. Physiol. 14, 101-120
40. Brown PR, Agarwal RP, Gell J & Parks RE (1972) Comp. Biochem. Physiol. 43B, 891-904
41. Brown GK & O'Sullivan WJ (1977) Biochem. Pharmacol. 26, 1947-1950
42. Burke WG, Chen S-H, Scott CR & Amman AJ (1977) J. Cell. Physiol. 92, 109-114
43. Burridge PW, Paetku V & Henderson JF (1977) J. Immunol. 119, 675-678
44. Carapella-De Luca E, Aiuti F, Lucarelli P et al. (1978) J. Pediat. 93, 1000-1003
45. Carson DA & Seegmiller JE (1976) J. Clin. Invest. 57, 274-282
46. Carson DA, Kaye J & Seegmiller JE (1977) Proc. Natl. Acad. Sci. USA 74, 5677-5681
47. Carson DA, Kaye J, Matsumoto S et al. (1979) Proc. Natl. Acad. Sci. 76, 2430-2433
48. Carson DA, Kaye J & Seegmiller JE (1979) In: Inborn errors of immunity and phagocytosis (Eds. F. Güttler, JWT Seakins & RA Harkness) pp. 129-135. MTP Press
49. Carson DA, Kaye J & Wasson DB (1980) J. Immunol. 124, 8-12
50. Castles JJ, Gershwin ME, Saito W et al. (1977) Dev. Comp. Immunol. 1, 165-174
51. Cartier PH & Hamet MA (1974) CR Acad. Sci. (Paris) 279, 883-886
52. Cartier PH (1980) J. Biol. Chem. 255, 4574-4582
53. Chan T-S (1978) Cell 14, 523-530
54. Chan T-S (1979) Proc. Natl. Acad. Sci. USA 76, 925-929
55. Chandra P, Chanana AD & Joel DD (1980) Proc. Soc. Exp. Biol. Med. 163, 291-295
56. Chang CH, Brockman RW & Bennett LL (1980) J. Biol. Chem. 255, 2366-2371
57. Chapman ML, Zaun MR & Gracy RW (1981) Biochem. Biophys. Res. Comm. 98, 303-310
58. Chechick BE, Rao J, Greaves MF & Hoffbrand AV (1980) Leukemia Res. 4, 343-349
59. Chechick BE, Schrader WP & Minowada J (1981) J. Immunol. 126, 1003-1007
60. Chen JJ & Jones ME (1976) Arch. Biochem. Biophys. 176, 82-90
61. Chen JJ & Jones ME (1979) J. Biol. Chem. 254, 2697-2704
62. Chen S-H, Ochs HD, Scott CR & Giblett ER (1979) Clin. Immunol. Immunopathol. 13, 156-160
63. Cohen A, Doyle D, Martin DW & Amman AJ (1976) N. Engl. J. Med. 295, 1449-1454
64. Cohen A, Hirschhorn R, Horowitz SD et al. (1978) Proc. Natl. Acad. Sci. USA 75, 472-476
65. Cohen A, Gudas LJ, Amman AJ et al. (1978) J. Clin. Invest. 61, 1405-1409
66. Cohen A, Mansour A, Dosch H-M & Gelfand EW (1980) Clin. Immunol. Immunopathol. 15, 245-250
67. Cohen A, Lee JWW, Dosch H-M & Gelfand EW (1980) J. Immunol. 125, 1578-1582
68. Cohen MB, Maybaum J & Sadee W (1981) J. Biol. Chem. 256, 8713-8717
69. Coleman MS, Donofrio J, Hutton JJ et al. (1978) J. Biol. Chem. 253, 1619-1626
70. Coleman MS, Greenwood MF, Hutton JJ et al. (1978) Blood 52, 1125-1131
71. Collier KJ & Ma PF (1980) Int. J. Biochem. 12, 659-662
72. Cooper EL (1977) TIBS 2, 130-132
73. Cowan MJ, Cashman D & Ammann AJ (1981) Biochem. Pharmacol. 30, 2651-2656

74. Cox RP, Krauss MR, Balis ME et al. (1974) Exp. Cell Res. 88, 289-294
75. Creasey WA & Handschumacher RE (1961) J. Biol. Chem. 236, 2058-2063
76. Daddona PE & Kelley WN (1980) Mol. Cell. Biochem. 29, 91-101
77. Danks MK & Scholar EM (1979) Biochem. Pharmacol. 28, 2733-2738
78. Danks MK & Scholar EM (1981) Int. J. Biochem. 13, 1261-1264
79. De Abreu RA, Van Baal JM, Bakkeren JAJM et al. (1982) J. Chromatogr. 227, 45-52
80. Debattisse M & Buttin G (1977) Som. Cell Gen. 3, 497-511
81. De Jong B, Anders GJPA, Van der Meer IH & Zijlstra J (1976) J. Immunol. Meth. 12, 91-102
82. Denman AM (1978) Brit. Med. J. ii, 980-982
83. Dent CE & Philpott GR (1954) Lancet i, 182-185
84. Dietz AA & Czebotar V (1977) Cancer Res. 37, 419-426
85. Donofrio J, Coleman MS, Hutton JJ et al. (1978) J. Clin. Invest. 62, 884-887
86. Dornand J, Bonnafous JC & Mani JC (1980) FEBS 118, 255-258
87. Dosch H-M, Mansour A, Cohen A et al. (1980) Nature 285, 494-496
88. Dowell BL, Segars JH & Metzgar RS (1981) Cancer Res. 41, 343-345
89. Drach JC, Thomas MA, Barnett JW et al. (1981) Science 212, 549-551
90. Duhm J (1974) Biochim. Biophys. Acta 343, 89-100
91. Dyminski JW, Daoud A, Lampkin BC et al. (1979) Clin. Immunol. Immunopathol. 14, 307-326
92. Edelson PJ & Schwaber J (1979) Science 205, 520-521
93. Edwards NL, Magilavy DB, Cassidy JT & Fox IH (1978) Science 201, 628-630
94. Edwards NL, Gelfand EW, Biggar D & Fox IH (1978) J. Lab. Clin. Med. 91, 736-749
95. Edwards NL, Gelfand EW, Burk L et al. (1979) Proc. Natl. Acad. Sci. USA 76, 3473-3476
96. Edwards NL, Cassidy JT & Fox IH (1980) Clin. Immunol. Immunopathol. 17, 76-88
97. Fahey KJ, Outteridge PM & Burrells C (1980) Austr. J. Exp. Biol. Med. Sci. 58, 571-583
98. Fauci AS (1981) J. Am. Med. Ass. 246, 2567-2572
99. Ferrante A & Thong YH (1980) J. Immunol. Meth. 34, 279-285
100. Fishbein WN, Ambrustmaker NW & Griffin JL (1978) Science 200, 545-548
101. Fishbein WN, Davis JI, Winkert JW & Fishbein JD (1980) Biochem. Med. 24, 130-142
102. Fishbein WN, Davis JI, Winkert JW & Strong DM (1981) Biochem. Med. 26, 377-386
103. Forsdyke DR (1967) Biochem. J. 105, 679-684
104. Fox IH & Kelley WN (1971) Ann. Int. Med. 74, 424-433
105. Fox IH & Kelley WN (1972) J. Biol. Chem. 247, 2126-2131
106. Fox IH, Keystone EC, Gladman DD et al. (1975) Immunol. Comm. 4, 419-427
107. Fox IH, Andres CM, Gelfand EW & Biggar D (1977) Science 197, 1084-1086
108. Fox IH, Lacroix S, Planet G et al. (1977) Medicine 56, 515-526
109. Fox IH & Kelley WN (1978) Ann. Rev. Biochem. 16, 655-686
110. Fox IH, Burk L, Planet G et al. (1978) J. Biol. Chem. 253, 6794-6800
111. Fox IH, Kaminska J, Wortmann RL & Halsey DL (1979) J. Lab. Clin. Med. 94, 52-59
112. Fox IH, Kaminska J, Edwards NL et al. (1980) Biochem. Gen. 18, 221-234
113. Fox IH (1981) Metabolism 30, 616-634

114. Fox RM, Wood MH & O'Sullivan WJ (1971) J. Clin. Invest. 50, 1050-1060
115. Fritzson P (1978) Advanc. Enzyme Reg. 16, 43-61
116. Fyfe JA, Miller RL & Krenitsky TA (1973) J. Biol. Chem. 248, 3801-3809
117. Ganeshaguru K. Lie N, Llewelin P et al. (1981) Leuk. Res. 5, 215-222
118. Garcia RC, Leoni P & Allison AC (1977) Biochem. Biophys. Res. Comm. 77, 1067-1073
119. Gelfand EW, Dosch HM, Biggar WD & Fox IH (1978) J. Clin. Invest. 61, 1071-1080
120. Gelfand EW, Lee JJ & Dosch HM (1979) Proc. Natl. Acad. Sci. USA 76, 1998-2002
121. Ghangas G & Reem GH (1979) J. Biol. Chem. 254, 4233-4237
122. Giblett ER, Anderson JE, Cohen F et al. (1972) Lancet ii, 1067-1069
123. Giblett ER, Ammann AJ, Sandman R et al. (1975) Lancet i, 1010-1013
124. Giblett ER (1981) In: The lymphocyte (Eds. KW Sell & WV Miller), pp. 123-134. Alan R. Riss, New York
125. Green H & Chan T-S (1973) Science 182, 836-837
126. Green CD & Martin DW (1973) Proc. Natl. Acad. Sci. USA 70, 3698-3702
127. Gudas LJ, Ullman B, Cohen A & Martin DW (1978) Cell 14, 531-538
128. Gudas LJ, Zannis VI, Clift SM et al. (1978) J. Biol. Chem. 253, 8916-8924
129. Hallak GJ & Wilkinson JH (1977) Enzyme 22, 361-369
130. Harkrader RJ, Boritsky T & Jackson RL (1981) Biochem. Pharmacol. 30, 1099-1104
131. Harley EH & Losman MJ (1981) Int. J. Biochem. 13, 247-249
132. Harrap KR & Paine RM (1977) Advanc. Enzyme Reg. 15, 169-193
133. Haurani FI, Kardinal LG & Biermann WA (1978) J. Cell. Physiol. 95, 49-56
134. Hedeskov CJ (1968) Biochem. J. 110, 373-380
135. Henderson JF, Fraser JH & McCoy EE (1974) Clin. Biochem. 7, 339-358
136. Henderson JF, Brox L, Zombor G et al. (1977) Biochem. Pharmac. 26, 1967-1972
137. Henderson JF (1979) In: Physiological and regulatory functions of adenosine and adenine nucleotides (Eds. HP Baer & GI Drummond), pp. 315-322. Raven Press, New York.
138. Henderson JF, Scott FW & Lowe JK (1980) Pharmac. Ther. 8, 573-604
139. Henderson JF & Scott FW (1980) Pharmac. Ther. 8, 539-571
140. Henderson JF & Smith CM (1981) In: Nucleosides and cancer treatment (Eds. MHN Tattersall & RM Fox), pp. 208-217. Academic Press, Sydney
141. Hershfield MS & Seegmiller JE (1977) J. Biol. Chem. 252, 6002-6010
142. Hershfield MS, Snyder FF & Seegmiller JE (1977) Science 197, 1284-1287
143. Hershfield MS (1979) J. Biol. Chem. 254, 22-25
144. Hershfield MS, Kredich NM, Ownby DR et al. (1979) J. Clin. Invest. 63, 807-811
145. Hershfield MS & Kredich NM (1980) Proc. Natl. Acad. Sci. USA 77, 4292-4296
146. Hershfield MS (1981) J. Clin. Invest. 67, 696-701
147. Hershko A, Razin A & Mager J (1969) Biochim. Biophys. Acta 184, 64-76
148. Herzfeld A & Raper SM (1980) Cancer Res. 40, 744-750
149. Hill DL & Bennett LL (1969) Biochemistry 8, 122-130
150. Hirschhorn R, Grossman J & Weissmann G (1970) Proc. Soc. Exp. Biol. Med. 133, 1361-1365

151. Hirschhorn R & Levytska V (1974) Cell. Immunol. 12, 387-395
152. Hirschhorn R & Sela E (1977) Cell. Immunol. 32, 350-360
153. Hirschhorn R, Martiniuk F & Rosen FS (1978) Clin. Immunol. Immunopathol. 9, 287-292
154. Hirschhorn R, Vawter GF, Kirkpatrick JA & Rosen FS (1979) Clin. Immunol. Immunopathol. 14, 107-120
155. Hirschhorn R, Roegner V, Jenkins T et al. (1979) J. Clin. Invest. 64, 1130-1139
156. Hirschhorn R, Bajaj S, Borkowsky W et al. (1979) Cell. Immunol. 42, 418-423
157. Hirschhorn R, Roegner V, Rubinstein A & Papageorgiou P (1980) J. Clin. Invest. 65, 768-771
158. Hirschhorn R, Papageorgiou P, Kesarwala HH & Taft LT (1980) N. Engl. J. Med. 303, 377-380
159. Hirschhorn R & Ratech H (1980) Current topics in biological and medical research 4, 131-157
160. Hirschhorn R & Ratech H (1980) In: Primary immunodeficiencies. INSERM symp. no. 16 (Eds. M Seligman & WH Hitzig). pp. 237-250, Elsevier/North Holland
161. Hitzig WH, Dooren LJ & Vossen JM (1978) Springer Sem. Immunopathol. 1, 283-298
162. Hoffman DR, Cornatzer WE & Duerre JA (1979) Can. J. Biochem. 57, 56-65
163. Holmes EW, McDonald JA, McCord JM et al. (1973) J. Biol. Chem. 248, 144-150
164. Hoogenraad NJ & Lee DC (1974) J. Biol. Chem. 249, 2763-2768
165. Hooton JWL & Hoffbrand AV (1976) Brit. J. Haematol. 33, 527-537
166. Hovi T, Allison AC & Allsop J (1975) FEBS Lett. 55, 291-293
167. Hovi T, Smyth JF, Allison AC & Williams SC (1976) Clin. Exp. Immunol. 23, 395-403
168. Hovi T, Allison AC, Raivio KO & Vaheri A (1977) Ciba Foundation symposium 48, 225-248
169. Huguley CM, Bain JA, Rives S & Scoggins R (1959) Blood 14, 615
170. Huisman WH, Raivio KO & Becker MA (1979) J. Biol. Chem. 254, 12595-12602
171. Hunting D & Henderson JF (1978) Biochem. Pharmacol. 27, 2163-2169
172. Hunting D & Henderson JF (1981) Can. J. Biochem. 59, 723-727
173. Hunting D & Henderson JF (1981) Can. J. Biochem. 59, 830-837
174. Hunting D, Hordern J & Henderson JF (1981) Can. J. Biochem. 59, 838-847
175. Hutton JJ, Wiginton DA, Coleman MS et al. (1981) J. Clin. Invest. 68, 413-421
176. Ikezawa Z, Nishino T, Murakami K & Tsuchiya K (1978) Comp. Biochem. Physiol. 60B, 111-116
177. Ishii K & Green H (1973) J. Cell Sci. 13, 429-439
178. Ito K & Uchino H (1971) J. Biol. Chem. 246, 4060-4065
179. Ito K & Uchino H (1973) J. Biol. Chem. 248, 389-392
180. Ito K & Uchino H (1976) J. Biol. Chem. 254, 1427-1430
181. Ives DH & Durham JP (1970) J. Biol. Chem. 245, 2285-2294
182. Jackson RC, Morris HP & Weber G (1978) Br. J. Cancer 37, 701-713
183. Jackson RC, Goulding FJ & Weber G (1979) J. Natl. Cancer. Inst. 62, 749
184. Jackson RC, Ross DA, Harkrader RJ & Epstein J (1980) Cancer Treatm. Rep. 64, 1347-1353
185. Jenkins T, Rabson AR, Nurse GT et al. (1976) J. Pediat. 89, 732-736
186. Jones ME (1980) Ann. Rev. Biochem. 49, 253-279
187. Johnson SM, North ME, Asherson GL et al. (1977) Lancet 1, 168-170
188. Johnston JM & Kredich NM (1979) J. Immunol. 123, 97-103
189. Kamatani N & Carson DA (1981) Biochim. Biophys. Acta 675, 344-350
190. Kaminska JF & Fox IH (1980) J. Lab. Clin. Med. 96, 141-147

191. Kamoun P, Chanard J, Brani M & Funck-Brentano JL (1978) Clin. Sci. Mol. Med. 54, 595-601
192. Kay JE & Handmaker SD (1970) Exp. Cell Res. 63, 411-421
193. Kay JE, Ahem T, Lindsay VJ & Sampson J (1975) Biochim. Biophys. Acta 378, 241-250
194. Kay NMB (1979) Mech. Ageing Develop. 9, 39-59
195. Kefford RF & Fox RM (1982) Cancer Res. 42, 324-330
196. Kelley WN (1974) Enzyme 18, 161-175
197. Kelley WN, Holmes EW & Van der Weyden MB (1975) Arthritis Rheum. 18, 673-680
198. Kensler TW, Cooney DA, Jayaram HN et al. (1981) Anal. Biochem. 117, 315-319
199. Kim BK, Cha S & Parks RE (1968) J. Biol. Chem. 243, 1763-1770
200. Klaushofer K, Mayer D, Hummel W & Von Mayerback H (1979) Enzyme 24, 77-84
201. Kozai Y, Sonoda S, Kobayashi S & Sugino Y (1972) J. Biochem. 71, 485-496
202. Kredich NM & Hershfield MS (1979) Proc. Natl. Acad. Sci. USA 76, 2450-2454
203. Krenitsky TA, Tuttle JV, Koszalka GW et al. (1976) J. Biol. Chem. 251, 4055-4061
204. Kruisbeek AM (1978) Thymus-dependent immune competence. Effects of ageing, tumour- and thymic humoral function. Thesis, State University of Utrecht
205. Krygier V & Momparler RL (1971) J. Biol. Chem. 246, 2752-2757
206. Kuttesch JF, Schmalstieg FC & Nelson JA (1978) J. Liq. Chromat. 1, 97-109
207. Lazary S, De Weck AL, Gerber H et al. (1973) Z. Immun. Forsch. 145, 361-375
208. Levine RL, Hoogenraad NJ & Kretchmer N (1974) Pediat. Res. 8, 724-734
209. Lesch M & Nyhan WL (1964) Am. J. Med. 36, 561-570
210. Lin AL & Elford HL (1980) J. Biol. Chem. 255, 8523-8528
211. Ling NR & Kay JE (1975) Lymphocyte stimulation. 2nd edition. North Holland, Amsterdam
212. Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) J. Biol. Chem. 193, 265-275
213. Lucas ZJ (1967) Science 156, 1237-1239
214. Lucas ZJ (1969) In: Proc. 3rd Ann. Leucocyte Culture conference, 1967, Iowa City, pp. 655-668. Appleton-Century-Crofts, New York.
215. Ludwig H, Kuzmits R, Pietschmann H & Müller MM (1979) Blut 39, 309-315
216. Lukey T & Snyder FF (1980) Can. J. Biochem. 58, 677-682
217. Lukey T & Snyder FF (1981) Comp. Biochem. Physiol. 69B, 547-551
218. Lum CL, Marr R, Plagemann PGW & Wohlhueter RM (1979) J. Cell. Physiol. 101, 173-200
219. Ma PF & Magers TA (1975) Int. J. Biochem. 6, 281-286
220. MacDermott RP, Tritsch GL & Formeister JF (1980) Clin. Exp. Immunol. 42, 303-307
221. Magnuson NS & Perryman LE (1979) J. Clin. Invest. 64, 89-101
222. Magnuson NS & Perryman LE (1980) Comp. Biochem. Physiol. 67B, 205-211
223. Magnuson NS, McConnell LA & Perryman LE (1981) Comp. Biochem. Physiol. 70B, 279-288
224. Manery JF & Dryden EE (1979) In: Physiological and regulatory functions of adenosine and adenine nucleotides (Eds. HP Baer & GI Drummond), pp. 323-339. Raven Press, New York
225. Marcolongo R, Pompucci G & Michelli V (1975) Experientia 31, 1139
226. Marone G, Plaut M & Lichtenstein LM (1980) La ricerca Clin. Lab. 10, 303-312
227. Martin DW & Gelfand EW (1981) Ann. Rev. Biochem. 50, 845-877

228. May RA & Hoffee P (1979) Arch. Biochem. Biophys. 193, 398-406
229. McGuire T, Pollara B, Moore JJ & Poppie MJ (1976) Infec. Immun. 13, 995-997
230. McGuire T, Perryman LE & Banks KL (1981) Vet. Immunol. Immunopathol. 2, 101-109
231. McHaffie RA & Wang CH (1967) Blood 29, 640-646
232. Meyer J & Nygaard P (1979) Leukemia Res. 3, 211-216
233. Meyskens RL & Williams HE (1971) Biochim. Biophys. Acta 240, 170-179
234. Miller RL, Adamczyk DL, Miller WH et al. (1979) J. Biol. Chem. 254, 2346-2352
235. Mitchell BS, Meijas E, Daddona PE & Kelley WN (1978) Proc. Natl. Acad. Sci. 75, 5011-5014
236. Mitchell BS, Koller CA & Heyn R (1980) Blood 56, 556-559
237. Mitchell BS & Kelley WN (1980) Ann. Int. Med. 92, 826-831
238. Moore EC & Hurlbert RB (1966) J. Biol. Chem. 241, 4802-4809
239. Müller MM, Pischek G, Scheiner O et al. (1979) Blut 38, 447-455
240. Müller MM, Pischek G, Scheiner O et al. (1980) Blut 40, 137-145
241. Müller WEG, Zahn RK, Arendes J et al. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 1287-1295
242. Munch-Petersen B, Tyrsted G & Dupont B (1973) Exp. Cell Res. 79, 249-256
243. Munch-Petersen B & Tyrsted G (1977) Biochim. Biophys. Acta 478, 364-375
244. Murray AW (1971) Ann. Rev. Biochem. 40, 811-826
245. Musick WDL (1981) CRC Crit. Rev. Biochem. 11, 1-34
246. Nelson JA, Kuttesch JF, Goldblum RM et al. (1979) In: Physiolocal and regulatory functions of adenosine and adenine nucleotides (Eds. HP Baer & GI Drummond), pp. 417-427. Raven Press, New York
247. Newby AC (1980) Biochem. J. 186, 907-918
248. Newby AC & Holmquist CA (1981) Biochem. J. 200, 399-403
249. Nishida Y, Kamatani N, Tanimoto K & Akaoka I (1979) Agent Action 9, 549-552
250. Nishida Y, Okudaira K, Tanimoto K & Akaoka I (1980) Exp. Hemat. 8, 593-598
251. Nishida Y, Akaoka I & Horiuchi Y (1981) Biochem. Med. 26, 387-394
252. Nordeen SK & Young DA (1977) J. Biol. Chem. 252, 5324-5331
253. North ME, Newton CA & Webster ADB (1980) Clin. Exp. Immunol. 42, 523-529
254. Ochs UH, Chen S-H, Ochs HD et al. (1979) J. Immunol. 122, 2424-2430
255. O'Leary JJ, Mehta C, Hall DJ & Rosenberg A (1980) Cell Tissue Kin. 13, 21-32
256. Osborne WRA, Chen S-H, Giblett ER et al. (1977) J. Clin. Invest. 60, 741-746
257. Osborne WRA (1980) J. Biol. Chem. 255, 7089-7092
258. Osborne WRA, Sullivan JL & Scott CR (1980) Immunol. Comm. 9, 257-267
259. Osborne WRA (1981) TIBS 6, 80-81
260. Paglia DE & Valentine WN (1975) J. Biol. Chem. 250, 7973-7979
261. Parkman R, Gelfand EW, Rosen FB et al. (1975) N. Engl. J. Med. 292, 714-719
262. Parks RE, Stoeckler JD, Cambor C et al. (1981) In: Molecular actions and targets for cancer chemotherapeutic agents, pp. 229-252. Academic Press, Inc.
263. Patterson ARP, Kolassa N & Cass CE (1981) Pharmac. Ther. 12, 515-536
264. Pausch J, Keppler D & Decker K (1972) Biochim. Biophys. Acta 258, 395-403
265. Payne MR, Dancis J, Berman PH & Balis ME (1970) Exp. Cell Res. 59, 489-490

266. Pegararo L & Bernengo MG (1971) Exp. Cell Res. 68, 283-290
267. Peters GJ & Veerkamp JH (1979) Int. J. Biochem. 10, 885-888
268. Peters GJ, Oosterhof A & Veerkamp JH (1980) Arch. Int. Physiol. Biochem. 88, B241-242
269. Peters GJ, Oosterhof A & Veerkamp JH (1981) Int. J. Biochem. 13, 445-455
270. Peters GJ, Oosterhof A & Veerkamp JH (1981) Int. J. Biochem. 13, 577-583
271. Peters GJ, Oosterhof A & Veerkamp JH (1982) Int. J. Biochem. in press
272. Peters GJ & Veerkamp JH (1982) Vet. Immunol. Immunopathol., in press
273. Peters GJ, Oosterhof A & Veerkamp JH (1982) Biol. Neon., in press
274. Peters GJ, Oosterhof A & Veerkamp JH (1982) Biochim. Biophys. Acta, submitted for publication
275. Peters GJ, Oosterhof A & Veerkamp JH (1982) Eur. J. Biochem., submitted for publication
276. Peters GJ, Oosterhof A & Veerkamp JH (1982) Comp. Biochem. Physiol., in press
277. Plagemann PGW & Wohlhueter RM (1980) Current topics in membranes and transport 14, 225-230
278. Planet G & Fox IH (1976) J. Biol. Chem. 251, 5839-5844
279. Polmar SH, Wetzler EM, Stern RC & Hirschhorn R (1975) Lancet ii, 743-746
280. Polmar SH, Stern RC, Schwartz AL et al. (1976) N. Eng. J. Med. 295, 1337-1343
281. Polmar SH (1980) Seminars Hematol. 17, 30-43
282. Poplack DG, Sallan SE, Rivera G et al. (1981) Cancer Res. 41, 3343-3346
283. Poplack DG, Blatt J & Reaman G (1981) Cancer Res. 41, 4821-4823
284. Prentice HG, Smyth JF, Ganeshaguru K et al. (1980) Lancet ii, 170-172
285. Raisonnier A, Bouma M-E, Salvat C & Infante R (1981) Eur. J. Biochem. 118, 565-569
286. Raivio KO & Hovi T (1977) Advances Exp. Med. Biol. 76A, 448-455
287. Raivio KO & Hovi T (1978) Exp. Cell. Res. 116, 75-83
288. Raivio KO (1980) Eur. J. Pediat. 135, 13-20
289. Raivio KO, Lazar CS & Becker MA (1981) Biochim. Biophys. Acta 678, 58-64
290. Raivio KO, Lazar CS, Krumholz HR & Becker MA (1981) Biochim. Biophys. Acta 678, 51-57
291. Ramot B, Brok-Simoni F, Barnea N et al. (1977) Brit. J. Haematol. 36, 67-70
292. Rapoport E & Zamecnik PC (1978) Proc. Natl. Acad. Sci. 75, 1145-1147
293. Ratech H, McCarthy GW & Meuwissen HJ (1980) Comp. Biochem. Physiol. 67B, 333-335
294. Ratech H & Hirschhorn R (1981) Clin. Chim. Acta 115, 341-347
295. Ratech H, Thorbecke GJ & Hirschhorn R (1981) Clin. Immunol. Immunopathol. 21, 119-127
296. Ratech H, Thorbecke GJ, Meredith G & Hirschhorn R (1981) Enzyme 26, 74-84
297. Reaman GH, Blatt J & Poplack DG (1981) Blood 58, 330-332
298. Recker DP, Edwards NL & Fox IH (1980) J. Lab. Clin. Med. 95, 175-179
299. Reem GH (1974) J. Biol. Chem. 249, 1696-1703
300. Reibel DK & Rovetto MJ (1978) J. Chromatogr. 161, 406-409
301. Reichard P (1978) Fed. Proc. 37, 9-14
302. Rich KC, Arnold WJ, Palella T & Fox IH (1979) Am. J. Med. 67, 172-176
303. Rich KC, Meijas E & Fox IH (1980) N. Eng. J. Med. 303, 973-977
304. Robbins DL & Gershwin ME (1978) Sem. Arthrit. Rheum. 7, 325-371

305. Roberts W, Kartha M & Sagone AL (1979) Radiation Res. 79, 601-610
306. Roth DG & Deuel TF (1974) J. Biol. Chem. 249, 297-301
307. Roth DG, Shelton E & Deuel TF (1974) J. Biol. Chem. 249, 291-296
308. Roux JM (1973) Enzyme 15, 361-377
309. Rowe M & Johnson SM (1979) Biochem. Soc. Trans. 7, 997
310. Rowe M, De Gast GL, Platts-Mills TAF et al. (1980) Clin. Exp. Immunopathol. 39, 337-343
311. Rubinstein A, Hirschhorn R, Sicklick M & Murphy RA (1979) N. Eng. J. Med. 300, 387-392
312. Russell NH, Prentice HG, Lee N et al. (1981) Brit. J. Haematol. 49, 1-9
313. Sagone AL, LoBuglio A & Balzercak P (1974) Cell. Immunol. 14, 443-452
314. Sakura N, Usui T & Ito K (1980) Hiroshima J. Med. Sci. 29, 195-201
315. Sandman R, Ammann AJ, Groce C & Wara DW (1977) Clin. Immunol. Immunopathol. 8, 247-253
316. Sasaki R, Bollum FJ & Goldschneider I (1980) J. Immunol. 125, 2501-2503
317. Savage B & Spencer N (1977) Biochem. J. 167, 703-710
318. Schmalstieg FC, Nelson JA, Mills GC et al. (1977) J. Pediat. 91, 48-51
319. Schmalstieg FC, Mills GC, Nelson JA et al. (1978) J. Pediat. 93, 597-603
320. Scholar EM & Calabresi P (1973) Cancer Res. 33, 94-103
321. Scholar EM, Brown PR, Parks RE & Calabresi P (1973) Blood 41, 927
322. Scholar EM, Rashidian M & Heidrick ML (1980) Mech. Ageing Dev. 12, 323-329
323. Schwartz AL, Stern EC & Polmar SH (1978) Clin. Immunol. Immunopathol. 9, 499-505
324. Schwarzmeier J, Moser K & Rainer H (1972) Klin. Wschr. 50, 871-874
325. Seegmiller JE, Rosenbloom FM & Kelley WN (1967) Science 155, 1682-1684
326. Seegmiller JE (1978) In: The year in metabolism, 1977 (Ed. N Freinkel), pp. 253-325. Plenum Press, New York
327. Seegmiller JE (1980) Brit. J. Ann. Rheum. Dis. 39, 103-117
328. Segel IH (1976) Biochemical calculations, 2nd edition. John Wiley & Sons, Inc., New York
329. Shambaugh GE (1979) Am. J. Clin. Nutr. 32, 1290-1297
330. Shoaf WT & Jones ME (1973) Biochemistry 12, 4039-4051
331. Siaw MFE, Mitchell BS, Koller CA et al. (1980) Proc. Natl. Acad. Sci. USA 77, 6157-6161
332. Siegenbeek van Heukelom IH, Staal GEJ, Stoop JW & Zegers BJM (1976) Clin. Chim. Acta 72, 117-124
333. Simmonds HA, Sahota A, Potter CF & Cameron JS (1978) Clin. Sci. Mol. Med. 54, 579-584
334. Simmonds HA, Panayi GS & Corrigall V (1978) Lancet i, 60-63
335. Simpkins H, Stanton A & Davis BA (1981) Cancer Res. 41, 3107-3110
336. Skaper SD, Willis RC & Seegmiller JE (1976) Science 193, 587-588
337. Skupp S, Vugrek G & Ayvazian JH (1979) Biochem. Pharmacol. 28, 3323-3331
338. Smith JW, Steiner AL & Parker CW (1971) J. Clin. Invest. 50, 442-448
339. Smith CM & Henderson JF (1976) Can. J. Biochem. 54, 341-349
340. Smith CM, Belch A & Henderson JF (1980) Biochem. Pharmacol. 29, 1209-1210

341. Smith GP, Shah T, Webster ADB & Peters TJ (1981) Clin. Exp. Immunol. 46, 321-326
342. Smyth JF, Poplack DG, Holiman BJ et al. (1978) J. Clin. Invest. 62, 710-712
343. Snyder FF, Mendelsohn J & Seegmiller JE (1976) J. Clin. Invest. 58, 654-666
344. Snyder FF, Hershfield MS & Seegmiller JE (1978) Cancer Res. 38, 2357-2362
345. Snyder FF & Lukey T (1980) Adv. exp. med. biol. 122B, 259-264
346. Sperling O, Boer P, Brosh S et al. (1978) Enzyme 23, 1-9
347. Sperling O, Brosh S, Boer P et al. (1979) Biomed. 31, 20-23
348. Staal GEJ, Stoop JW, Zegers BJM et al. (1980) J. Clin. Invest. 65, 103-108
349. Stoeckler JD, Agarwal RP, Agarwal KC et al. (1978) Biochemistry 17, 278-283
350. Stoeckler JD, Cambor C & Parks RE (1980) Biochemistry 19, 102-107
351. Stoop JW, Zegers BJM, Hendrickx GFM et al. (1977) N. Eng. J. Med. 296, 651-655
352. Stoop JW, Zegers BJM, Heynen CJ et al. (1980) In: Primary Immunodeficiencies, INSERM symposium no. 16 (Eds. M. Seligman & WH Hitzig), pp. 301-311. Elsevier/North Holland, Amsterdam
353. Sullivan JL, Osborne WRA & Wedgwood RJ (1977) Brit. J. Haematol. 37, 159-160
354. Switzer RL (1971) J. Biol. Chem. 246, 2447-2458
355. Switzer RL & Sogin DC (1973) J. Biol. Chem. 248, 1063-1079
356. Szeinsberg A, Gavendo S & Cahane D (1969) Lancet i, 315
357. Taheri MR, Wickremasinghe RG & Hoffbrand AV (1981) Biochem. J. 194, 451-461
358. Taheri MR, Wickremasinghe RG & Hoffbrand AV (1981) Biochem. J. 196, 225-235
359. Tatibana M & Shigesada K (1972) Biochem. Biophys. Res. Comm. 46, 491-497
360. Tatibana M (1978) In: Handbook of experimental pharmacology, vol. 51 (Eds. WN Kelley & JM Weiner), pp. 125-154. Springer Verlag, Berlin
361. Tattersall MHN, Lavoie A, Ganeshaguru K et al. (1975) Eur. J. Clin. Invest. 5, 191-202
362. Tattersall MHN, Ganeshaguru K & Hoffbrand AV (1975) Biochem. Pharmacol. 24, 1495-1498
363. Tax WJM, Veerkamp JH & Trijbels JMF (1976) Comp. Biochem. Physiol. 54B, 209-212
364. Tax WJM, Veerkamp JH, Trijbels JMF & Schretlen EDAM (1976) Biochem. Pharmacol. 25, 2025-2032
365. Tax WJM & Veerkamp JH (1977) Clin. Chim. Acta 78, 209-216
366. Tax WJM & Veerkamp JH (1978) Comp. Biochem. Physiol. 59B, 219-222
367. Tax WJM (1978) The interrelationship of purine and pyrimidine metabolism in mammalian blood cells. Thesis, University of Nijmegen.
368. Tax WJM & Veerkamp JH (1978) Comp. Biochem. Physiol. 61B, 439-441
369. Tax WJM & Veerkamp JH (1979) Biochem. Pharmacol. 28, 829-831
370. Tax WJM, Peters GJ & Veerkamp JH (1979) Int. J. Biochem. 10, 7-10
371. Tedde A, Balis ME, Schonberg R & Trotta PP (1979) Cancer Res. 39, 3044-3050
372. Tedde A, Balis ME, Ikehara S et al. (1980) Proc. Natl. Acad. Sci. USA 77, 4899-4903
373. Thompson LF, Boss GR, Spiegelberg HL et al. (1979) J. Immunol. 123, 2475-2478
374. Thuillier L, Garreau F & Cartier PH (1981) Eur. J. Immunol. 11, 788-794

375. Torrance JD & Whittaker DA (1981) Enzyme 26, 64-73
376. Traut TW & Jones ME (1977) Biochem. Pharmacol. 26, 2291-2296
377. Tritsch GL & Niswander PW (1981) Biochem. Med. 26, 185-190
378. Tritsch GL & Niswander PW (1981) Immunol. Comm. 10, 1-8
379. Trotta PP, Ahland MP, Brown GF & Balis ME (1978) Mol. Pharmacol. 14, 199-209
380. Trotta PP, Peterfreund RA, Schonberg R & Balis ME (1979) Biochemistry 18, 2953-2959
381. Tsuchiya S. Arai N, Kudo M et al. (1979) Tokuhu J. Exp. Med. 128, 251-258
382. Tung R, Silber R, Quagliata F et al. (1976) J. Clin. Invest. 57, 756-761
383. Tyrsted G & Sartorelli AC (1968) Biochim. Biophys. Acta 155, 619-622
384. Tyrsted G, Munch-Petersen B & Cloos L (1973) Exp. Cell Res. 77, 415-427
385. Tyrsted G (1975) Exp. Cell Res. 91, 429-440
386. Uberti J, Lightbody JJ, Wolf JW et al. (1978) Clin. Immunol. Immunopathol. 10, 446-458
387. Uberti J, Lightbody JJ & Johnson RM (1979) J. Immunol. 123, 189-193
388. Ullman B, Gudas LJ, Cohen A & Martin DW (1978) Cell 14, 365-375
389. Ullman B, Gudas LJ, Clift SM & Martin DW (1979) Proc. Natl. Acad. Sci. USA 76, 1074-1078
390. Ullman B, Clift SM, Gudas LJ et al. (1980) J. Biol. Chem. 255, 8308-8314
391. Ullman B, Levinson BB, Hershfield MS & Martin DW (1981) J. Biol. Chem. 256, 848-852
392. Usher DC & Reiter H (1977) Cell 12, 365-370
393. Uusitalo RJ (1981) Histochem. J. 13, 525-534
394. Valentine WN, Fink K. Paglia DE et al. (1974) J. Clin. Invest. 54, 866-879
395. Valentine WN, Paglia DE, Tartaglia AP & Gilsanz F (1977) Science 195, 783-785
396. Van Belle H (1969) Biochim. Biophys. Acta 192, 124-132
397. Van den Berghe G, Bronfman M, Vanneste R & Hers HG (1977) Biochem. J. 162, 601-609
398. Van den Berghe G, Bontemps F & Hers HG (1980) Biochem. J. 188, 913-920
399. Van der Weyden MB & Kelley WN (1976) J. Biol. Chem. 251, 5448-5456
400. Van der Weyden MB & Bailey L (1978) Clin. Chim. Acta 82, 179-184
401. Van Laarhoven JPRM, Spierenburg GT, De Bruyn CHMM & Schretlen EDAM (1980) Adv. Exp. Med. Biol. 122B, 283-288
402. Van Oosterom R (1980) Humoral function of the human thymus. Studies on the effects of human thymic epithelial conditioned medium on T-lymphocyte reactivity. Thesis, State University of Utrecht
403. Veerkamp JH, Peters GJ & Tax WJM (1979) In: Models for the study of inborn errors of metabolism (Ed. FA Hommes), pp. 171-177. Elsevier, Amsterdam
404. Venner PM, Glazer RI, Blatt J et al. (1981) Cancer Res. 41, 4508-4511
405. Wada Y, Nishimura Y, Tanubu M et al. (1974) Tokohu J. Exp. Med. 113, 149
406. Warren KR & Glinos AD (1976) Fed. Proc. 35, 1732
407. Watts RP, Brendel K, Luthra MG & Kim HD (1979) Life Sci. 25, 1577-1582
408. Watts RWE, Perera YS, Allsop J et al. (1979) Clin. Exp. Immunol. 36, 355-363
409. Weber G (1977) N. Eng. J. Med. 296, 486-493 and 541-551

410. Weber G, Jackson RC, Williams JC et al. (1977) Adv. Enzyme Reg. 15, 53-77
411. Webster ADB, North M, Allsop J et al. (1978) Clin. Exp. Immunol. 31, 456-463
412. Wiginton DA, Coleman MS & Hutton JJ (1980) J. Biol. Chem. 255, 6663-6699
413. Willemot J, Martineau R, DesRosiers C et al. (1979) Life Sci. 25, 1215-1222
414. Wilson JM, Mitchell BS, Daddona PE & Kelley WN (1979) J. Clin. Invest. 64, 1475-1484
415. Wolberg G, Zimmerman TP, Hiemstra K et al. (1975) Science 187, 957-959
416. Wolberg G, Zimmerman TP, Duncan GS et al. (1978) Biochem. Pharmacol. 27, 1487-1495
417. Wong PCL & Murray AW (1969) Biochemistry 8, 1608-1614
418. Wood AW & Seegmiller JE (1973) J. Biol. Chem. 248, 138-143
419. Wood AW, Astrin KH, McCrae ME & Becker MA (1973) Fed. Proc. Fed. Am. Soc. Exp. Biol. 32, 652
420. Wortmann RL, Andres C, Kaminska J et al. (1979) Arthrit. Rheum. 22, 524-531
421. Wortmann RL, Mitchell BS, Edwards NL & Fox IH (1979) Proc. Nat. Acad. Sci. USA 76, 2434-2437
422. Wung WE & Howell SB (1980) Clin. Chem. 26, 1704-1708
423. Wyngaarden JB & Holmes JR (1977) In: Purine and pyrimidine memetabolism; Ciba Foundation symposium 48, pp. 43-64
424. Yamada Y, Goto H & Ogasawara N (1980) Biochim. Biophys. Acta 616, 199-207
425. Yeh GC & Phang JMP (1981) Biochem. Biophys. Res. Comm. 103, 118-124
426. Yen RCK, Adams WB, Lazar C & Becker MA (1978) Proc. Nat. Acad. Sci. USA 75, 482-485
427. Yip LC & Balis ME (1975) Biochem. Biophys. Res. Comm. 63, 722-729
428. Yip LC, Tedde A & Balis ME (1980) Biochem. Pharmacol. 29, 2888-2890
429. Zannis V, Doyle D & Martin DW (1978) J. Biol. Chem. 253, 504-510
430. Ziegler JB, Lee GH, Van der Weyden MB et al. (1980) Arch. Dis. Childhood 55, 452-457
431. Zimmerman TP, Schmitges CJ, Wolberg G et al. (1980) Proc. Nat. Acad. Sci. USA 77, 5639-5643
432. Zimmerman TP, Wolberg G, Duncan GS & Elion GB (1980) Biochemistry 19, 2252-2259

Godefridus (Frits) Johannes Peters werd op 19 april 1952 geboren te Helden. Na het behalen van het Mulo-A diploma in 1967 bezocht hij het Blariacumcollege te Venlo-Blerick, waar hij in 1970 het HBS-B diploma behaalde. In hetzelfde jaar werd gestart met de studie biologie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen B4 (biologie en scheikunde) werd in september 1973 afgelegd. De doctoraalstudie omvatte de bijvakken genetica en exobiologie en het hoofdvak biochemie. In januari 1977 werd het doctoraalexamen biologie behaald (met onderwijsaantekening). Het hier beschreven onderzoek werd verricht op het biochemisch laboratorium van de Mesche Faculteit onder leiding van Prof. Dr. J.H. Veerkamp en gesubsidieerd uit de Universitaire Onderzoeks Pool van de Katholieke Universiteit van augustus 1977 tot november 1981. Gedurende deze tijd werd ook een bijdrage geleverd aan het onderwijs aan studenten medicijnen en tandheelkunde.

In 1978 is hij getrouwd met Marja Delahaije.

STELLINGEN

NIJMEGEN, 4 JUNI 1982

G.J. PETERS

1. Aktiviteitsmetingen en kinetische studies in extracten kunnen nuttige informatie geven over het metabolisme in intacte cellen.

Arch JRS & Newsholme EA (1978) Biochem. J. 174, 965-977
Snyder FF & Lukey T (1982) Biochim. Biophys. Acta 696, 299-307
Dit proefschrift

2. Remming van de thymidine incorporatie hoeft niet altijd samen te gaan met een remming van de DNA synthese en celgroei.

Carson DA & Seegmiller JE (1976) J. Clin. Invest. 57, 274-282
Drach JC, Thomas MA, Barnett JW et al. (1981) Science 212, 549-551

3. Als enzymaktiviteiten in geïsoleerde intacte cellen op eiwitbasis worden uitgedrukt, dient ook de eiwitinhoud van de cel vermeld te worden.

4. Aangezien ribonucleotide reductase niet alleen gereguleerd wordt door deoxyATP, zou ook de concentratie van andere regulerende nucleotiden beschouwd moeten worden in studies waarbij de resultaten verklaard worden door de accumulatie van deoxyATP.

5. Op grond van de resultaten van Tattersall en medewerkers en van Munch-Petersen en medewerkers mag niet geconcludeerd worden dat de deoxyATP concentratie in lymfocyten na PHA-stimulatie zou toenemen.

Munch-Petersen B, Tyrsted G & Dupont B (1973) Exp. Cell Res. 79, 249-256
Tattersall MHN, Lavoie A, Ganeshaguru K et al. (1975) Eur. J. Clin. Invest. 5, 191-202
Dit proefschrift, hoofdstuk 9

6. De rol van adenosine deaminase bij de superoxide vorming van macrofagen van gezonde en immunodeficiënte personen verdient nader onderzoek.

Tritsch GL & Niswander PW (1981) Biochem. Med. 26, 185-190
Yagawa K & Okamura J (1981) Infect. Immun. 32, 394-397

7. De specificiteit van "specifieke" remmers neemt af naarmate er meer publikaties over verschijnen.

8. De conclusie dat de vertakte aminozuur oxidatie in spieren van gevaste ratten hoger is dan in die van gevoede ratten, is onvoldoende bewezen.

   Goldberg AL & Odessey R (1972) Am. J. Physiol. 223, 1384-1391
   Tischler ME & Goldberg AL (1980) Am. J. Physiol. 238, E480-E486

9. Het gebruik van bloed bij het kweken van microörganismen kan variaties geven, die het stellen van een diagnose onbetrouwbaar maken.

   Sundqvist G & Johansson E (1982) Scand. J. Dent. Res. 90, 29-36

10. Een van de weinige overeenkomsten tussen de meeste wetenschappelijke tijdschriften is de stijl waarin de 'Editors' hun beslissingen weergeven.

    TIBS (1981) 6, V

11. Als een artikel meer dan 4 à 5 auteurs heeft, is het onwaarschijnlijk dat al deze auteurs een evenredige bijdrage hebben geleverd bij de totstandkoming.

12. Karl May's boeken getuigen van een diep inzicht in de cultuur en psyche van de Indiaan en de Arabier en geven een getrouw beeld van hun natuurlijke omgeving.

13. Het feit dat "Limburger" zelfs de kolommen van het gezaghebbende tijdschrift 'Science' haalt, geeft deze kaas een bijzonder odeur.

    Science (1981) 213, 1238

14. Gezien de verschillen in purine- en pyrimidinemetabolisme in lymfocyten van mens en paard, zouden 'centauren' een interessant modelsysteem kunnen zijn.

15. Brood van de echte warme bakker is minstens even gezond als zogenaamd alternatief brood.

Mededeling van L. Peters, echte warme bakker

16. Over 5 jaar is het raadzaam voor een doctorandus van voor de 'Twee Fasen Structuur' dit expliciet in zijn sollicitatiebrief te vermelden; over 10 jaar geldt dit ook voor gepromoveerden.

17. Voordat men een paard bestijgt, dient men te weten dat teugels geen rempedaal en sporen geen gaspedaal zijn.